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Development of a Liver Gene Therapy
Strategy for Haemophilia B
with Lentiviral Vectors

*PhD thesis in fulfilment of the requirements of the Open University for
the degree of Doctor of Philosophy in Molecular and Cellular Biology*

17th December 2012

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“Eppur si muove” - “Yet it moves”
(G. Galilei)

DECLARATION

This thesis has been composed by myself and has not been used in any previous application for a degree. Throughout the text I use both 'I' and 'We' interchangeably. All the results presented here were obtained by myself, except for:

1) Large-scale vector manufacturing and some of the quality controls, which were performed in collaboration with MolMed s.p.a. (Milan, Italy) or Généthon (Evry, France)

2) Haemophilia B dogs maintenance, portal vein infusion and coagulation assays on canine samples, which were performed by Timothy Nichols and his team (University of North Carolina, Chapel Hill, USA)

3) Activated partial thromboplastin time and Bethesda assay on mouse samples, which were performed in collaboration with Patrizia Della Valle and Armando D'Angelo (San Raffaele, Scientific Institute, Milan, Italy)

4) Histopathology analysis, which was performed by Francesca Sanvito and Claudio Doglioni (San Raffaele Scientific Institute, Milan, Italy)

5) Fractionation and sorting of liver cell populations, T- and B-cell assays, which were performed in collaboration with Andrea Annoni and Maria Grazia Roncarolo (Telethon Institute for Gene Therapy, Milan, Italy)

6) Coagulation Factor IX delivery by integrase-defective lentiviral vectors, which was performed in the laboratory of Thierry VandenDriessche and Marinee Chuah (Free University of Brussels, Brussels, Belgium)

7) Integration site analysis, which was performed in collaboration with Cynthia Bartholomae and Manfred Schmidt (National Center for Tumour Diseases, Heidelberg, Germany)

Genotoxicity studies in mouse models sensitised to develop hepatocellular carcinoma were performed in collaboration with Marco Ranzani and Eugenio Montini (Telethon Institute for Gene Therapy, Milan, Italy). Since my contribution was minor in these studies, data are not shown in the "results" chapter, but are mentioned in the discussion, as they are relevant for the overall understanding of the described liver gene therapy strategy.

All sources of information are acknowledged by means of reference.

ABSTRACT

Lentiviral vectors (LVs) are attractive tools for liver gene therapy, by virtue of their ability to stably integrate in the genome of target cells and the absence of pre-existing humoral and cellular immunity against vector components in most humans. We have previously reported long-term phenotypic correction of haemophilia B and transgene-specific immune tolerance induction after a single intravenous administration of LVs in mice, provided that transgene expression is stringently targeted to hepatocytes. This is achieved by a combination of transcriptional control, mediated by a synthetic hepatocyte-specific promoter and post-transcriptional control obtained by including in the transgene sequences complementary to the haematopoietic-specific microRNA 142, which binds and targets for degradation any residual transgene mRNA expressed in antigen presenting cells of liver and spleen.

We have now evaluated this gene therapy strategy in a large animal model. Our results show long-term canine Factor IX (FIX) expression up to 0.5-1% of normal levels and clinical improvement (almost complete prevention of spontaneous bleedings) in two haemophilia B dogs (>3.5 years cumulative follow up), with mild acute toxicity and without long-term adverse effects nor anti-transgene immune responses. The use of codon-optimised and hyper-functional FIX transgenes increased the potency of LVs (in the pharmacological meaning of efficacy *per dose*) >15-fold, allowing correction of the disease phenotype at low vector doses in mice, thus improving the therapeutic index of the gene therapy and prompting us to test this improvement in the next treated dog. We have investigated additional improvements in the potency of LVs for liver gene therapy, such as the use of the baculovirus envelope protein gp64, which improves hepatocyte targeting and LV particles resistance to complement-mediated inactivation. We performed a quantitative analysis of LV biodistribution within the liver cell populations and show that Kupffer cells uptake most vector genomes, despite being a small fraction of the total cells, and limit hepatocyte transduction at low administered LV doses. However, pre-treatment with a single dose of a clinically used proteasome inhibitor prior to LV administration reduces this trapping effect and increases hepatocyte transduction and therapeutic efficacy up to 3-fold in haemophilia B mice. We provide evidence that liver gene therapy can establish long-term FIX expression and immune

tolerance in mice even in the presence of pre-existing anti-FIX antibody immunity. Since insertional mutagenesis is a concern for integrating vectors, we set out to explore the potential advantages of integrase-defective LVs (IDLVs) to express transgenes in the adult liver, in which hepatocytes turnover is slow. We show that, while not optimal for stable gene replacement therapy in their current design, IDLVs may represent a valuable strategy to induce stable antigen-specific tolerance by transient gene transfer and offer a treatment for immune-mediated diseases. On the other hand, since LV integration is preferable for efficient stable liver gene transfer, we stringently assessed the risk of oncogenesis associated to LV integration in *ad hoc* mouse models that are sensitised to develop hepatocellular carcinoma and found no detectable increase in carcinogenesis upon liver gene therapy with LVs.

Overall our results position LVs as a promising platform for liver gene therapy that may well complement other available vectors to address the different challenges posed by the presentation of haemophilia and its complications in different patients and clinical conditions and may conceivably offer a therapeutic option for lysosomal and metabolic diseases.

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ACRONYMS AND ABBREVIATIONS

AAV	adeno-associated virus
Ad	adenoviral vector
ADA-SCID	SCID due to adenosine deaminase deficiency
ALT	alanine aminotransferase
APC	antigen presenting cell
aPTT	activated partial thromboplastin time
AST	aspartate aminotransferase
BDD	B-domain deleted
BOEC	blood-outgrowth endothelial cells
BU	Bethesda units
cFIX	canine Factor IX
co-cFIX	codon-optimised cFIX
cPPT	central polypurine tract
CTL	cytotoxic T lymphocyte
CTS	central termination sequence
DC	dendritic cell
DIC	disseminated intravascular coagulation
DMSO	dimethyl sulfoxide
dsDNA	double-stranded DNA
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot
ET	enhanced transthyretin
FACS	fluorescence-activated cell sorting
FBS	foetal bovine serum
Fc	Fragment crystallisable
FDA	food and drug administration
FIX	Factor IX
FV	Factor V
FVII	Factor VII
FVIII	Factor VIII
FX	Factor X
gDNA	genomic DNA
GFP	green fluorescent protein
GMP	good manufacturing practice
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HDAd	helper-dependent Ad
hFIX	human Factor IX
HGF	hepatocyte growth factor
HIV	human immunodeficiency virus
HRP	horseradish peroxidase

HSPC	hematopoietic stem/progenitor cell
i.m.	intramuscular
i.v.	intravenous
IDLV	integrase-defective LV
IFA	incomplete Freund adjuvant
IFN	interferon
Ig	Immunoglobulin
IHL	intra-hepatic lymphocyte
IL	interleukin
IMDM	Iscoe's modified Dulbecco's medium
ITI	immune tolerance induction
ITR	inverted terminal repeat
KC	Kupffer cell
KO	knockout
LAL	limulus amebocyte lysate
LAM-PCR	linear amplification-mediated PCR
LSEC	liver sinusoidal endothelial cell
LTR	long terminal repeat
LV	lentiviral vector
MHC	major histocompatibility complex
MHC-I	MHC class-I
MHC-II	MHC class-II
MOI	multiplicity of infection
NHP	non-human primate
nPC	non-parenchymal cell
OT	ornithine transcarbamylase
OVA	ovalbumin
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
PC	parenchymal cell
PCR	polymerase chain reaction
pDC	plasmacytoid DC
PGK	phosphoglycerate kinase
polyA	polyadenylation signal
PT	prothrombin time
qPCR	quantitative PCR
RCL	replication-competent lentivirus
RV	gammaretroviral vector
SB	sleeping beauty
scAAV	self-complementary AAV
SCID	severe combined immunodeficiency
SEM	standard error of the mean
SIN	self-inactivating
ssRNA	single-stranded RNA

TFPI	tissue factor pathway inhibitor
TGF- β	transforming growth factor-beta
TLR	Toll-like receptor
TNF- α	tumour necrosis factor-alpha
Tregs	regulatory T cells
TT	thrombin time
TU	transducing unit
VCN	vector copy number
vg	vector genome
vp	vector particle
VSV-G	vesicular stomatitis virus glycoprotein G
VWF	von Willebrand factor
WBCT	whole-blood clotting time
wpre	woodchuck hepatitis virus post-transcriptional regulatory element
wt	wild-type
ZFN	zinc-finger nuclease

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1.INTRODUCTION

1.1 Gene Therapy

Gene therapy is an innovative approach to molecular medicine, based on the idea that the transfer of a gene into a target cell can counteract a disease. This idea stems from the universality of the genetic code, which enables a cell to “understand” any foreign gene¹.

Gene therapy was originally conceived to replace a defective function in a cell and treat genetic diseases. As molecular and cellular biology evolved and the pathophysiology of diseases were progressively elucidated, it was clear that many diseases have, at least in part, a genetic basis. Therefore it became apparent that gene therapy could be applied not only to monogenic diseases *strictu sensu*, but also to multigenic, multifactorial complex diseases, such as cardiovascular, neurodegenerative, infectious diseases and cancer. Scientists soon realised the enormous impact that gene therapy could have on medicine. The simple concept at the basis of gene therapy is the transfer of a classically defined gene or any regulatory nucleic acid in cells of human beings to cure or ameliorate a disease by instructing defective or new functions. However, this simple concept masks a stunning complexity, since mammalian cells and multicellular organisms have evolved physical, chemical and biological barriers to protect their own genetic information. To deliver the new gene (transgene) into a human tissue, a vehicle known as vector is needed in order to reach the target cells, pass the plasma membrane, enter the nucleus and appropriately direct expression of the gene, overcoming the immune defences outside and inside the cells (Figure I). Moreover, to make gene therapy acceptable for clinical administration, transgene delivery must be accomplished without causing adverse effects to patients. Since the first clinical trials in the 1990s, it took more than two decades to show convincing clinical benefits, a relatively short period, considering the significant hurdles and setbacks that had to be faced, undertaking such complex experimental therapies. Over 1,800 clinical trials have been conducted all over the world (www.wiley.com/legacy/wileychi/genmed/clinical), but only in recent years the therapeutic potential of gene therapy has become more widely appreciated throughout the scientific community²⁻⁵.

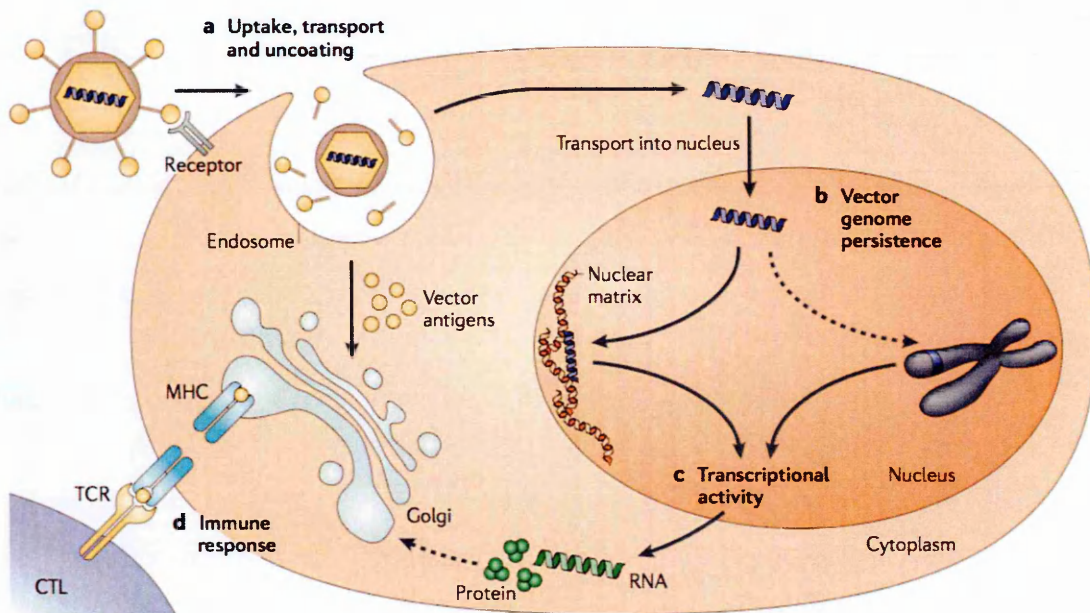


Figure 1: The major steps to gene transfer and potential barriers. (A) Uptake, transport and uncoating. Vectors bind to a cellular membrane and are internalised by various processes. Most uptake steps involve a ligand–receptor interaction. Once internalised, most vectors enter the endosome and undergo a complex set of reactions that can result in their full or partial degradation. Transport to the nucleus is also required for successful therapy. (B) Vector genome persistence. Once the vector reaches the nucleus, it can be further processed. Depending on the vector, the DNA can exist as an episomal molecule (and associate with the nuclear matrix) or it can be integrated (by covalent attachment) into the host chromosome. (C) Transcriptional activity and transgene persistence are dependent on many factors. (D) The immune response can limit the viability of the genetically modified cells and/or neutralize the transgene product. Adapted from Kay et al., *Nature Reviews Genetics*, 2011.

1.1.1 A brief history of gene therapy

From the first demonstration in the 1940s that transferring genomic DNA between strains of bacteria transformed the recipient phenotype into that of the donor phenotype⁶, many years elapsed before mammalian cells were successfully transformed with foreign DNA, although with extreme inefficiency⁷. As the techniques to transfer genetic material improved, gene transfer enabled the study of gene function as well as the production of recombinant proteins and it became clear that these tools might be used in humans to treat diseases.

The first ideas to develop gene therapies date back to the 1970s⁸. However efficient methods for gene transfer were lacking, because knowledge of “transforming” viruses preceded the recombinant DNA era and it was not clear how to modify these viruses to incorporate and express foreign genes. Some of the first gene therapy experiments were designed to treat blood diseases, by introducing the correct gene into bone marrow hematopoietic cells and re-infusing them into recipient mice⁹. The basis for these attempts was that transplantation of bone marrow hematopoietic cells were already being performed in the clinics and thus would facilitate clinical translation. These were the first examples of those protocols today referred to as *ex vivo* gene therapy (see 1.1.2). However, gene transfer technologies at that time could not allow the genetic modification of a significant number of cells. Because of this, attention focused to a specific type of haematopoietic disorders, the immune deficiencies, and in particular to the severe combined immune deficiency (SCID) due to the defective adenosine deaminase enzyme (ADA-SCID), whose gene was cloned in the 1980s. The rationale for choosing SCIDs as target diseases was based on the assumption that only a small fraction of corrected cells (potentially gaining selective growth advantage) could allow improvement of the immune-deficient phenotype, as compared to the high number of functional progenitors necessary for correcting other blood defects, such as anaemias¹⁰.

The first approved gene therapy trial started in 1990 and involved the transfer of the *ADA* gene in T cells from two young patients¹¹. In the same years, the pioneering work by Claudio Bordignon and his team led to the first human gene therapy clinical trial involving genetically modified bone marrow hematopoietic cells and T cells in patients affected by ADA-SCID¹². While the gene therapy resulted to be safe, enzyme activity was transient or subtherapeutic, mainly because of inefficient gene transfer and limited engraftment of the gene-modified cells. The 1990s were characterised by important technological developments and most of the virus-derived vectors that today are still in use in pre-clinical and clinical gene therapy were designed and generated. Since viruses had already evolved strategies to overcome the barriers to gene transfer into cells mentioned above (1.1), virus-derived vectors were conceived as the trojan horses that would carry the therapeutic genes into human cells (the most widely used viral vectors are discussed in more details in 1.3.4). After some of the technologies of genetic

manipulation were refined, a second set of clinical trials started in the late 1990s, for ADA-SCID and the X-linked SCID.

While the first encouraging results were emerging, a series of serious adverse events tuned down the enthusiasm and fuelled scepticism over gene therapy. Two of these major adverse events were due to host immune responses to a vector (upon systemic vector injection) and insertional mutagenesis caused by other vectors (in an *ex vivo* gene therapy trial). In 1999 Jesse Geslinger, a young boy with a relatively mild form of the nitrogen metabolism disorder ornithine transcarbamylase (OT) deficiency died on a gene therapy trial, after infusion to the liver of a viral vector carrying the OT transgene, causing a fatal inflammatory response¹³. This event was most probably caused by several interactions between the innate immune system and the vector delivered systemically. These interactions were insufficiently monitored in preclinical animal models. In the same years the first cases of leukaemias in gene therapy-treated SCID children were reported¹⁴. The young patients received infusion of their own hematopoietic stem and progenitor cells (HSPCs) genetically corrected with the functional gene of the interleukin-2 receptor γ chain, whose deficiency causes failure of lymphocytes to differentiate. The viral vector used to shuttle the gene inserted its genome close to a proto-oncogene and promoted its over-expression and subsequent aberrant T cells proliferation. This phenomenon, referred to as insertional mutagenesis, was observed with unexpectedly high frequency even in subsequent clinical trials for other immune deficiencies (see also 1.1.6)¹⁵⁻¹⁷. Both the host immune response and insertional mutagenesis still remain two major hurdles for gene therapy.

These adverse events soon hid the scientific and therapeutic progress in the press and gene therapy became perceived as a dangerous technology that did not live up the expectations that had raised¹⁸. Over-optimism in the initial years gave the way to disappointing results of lack of efficacy and unacceptable toxicities, such as in the above described trials. Regulatory authorities put gene therapies under intense scrutiny and threw the field into recession. However, this disillusionment forced gene therapists to better investigate the interactions between the vectors and both the immune system and genome of the host, to develop improved vectors and gene transfer protocols and to adopt more sensitive animal models. Overall, this turn of events taught not to underestimate the challenges faced by any novel therapy at the initial stage¹⁸. Only in

recent years the results of some successful clinical trials has renewed the promise of gene therapy.

Today some of the most successful examples of clinical gene therapy have been obtained in monogenic diseases. Several studies have now reported remarkable clinical improvements after gene therapy in patients with Leber's congenital amaurosis, a form of retinal blindness¹⁹⁻²¹. Affected patients received injections of the vector carrying the retinal pigment epithelium-specific 65 kDa protein gene necessary to reconstitute photoreceptor function directly into the eye, an immune-privileged site. In 2009 Aiuti *et al.* reported a 10-year follow up of ADA-SCID patients who received transplantation of autologous HSPCs modified with a vector carrying the functional ADA transgene, showing long-term ADA expression and therapeutic efficacy without adverse events²². The development of newer, more efficient and potentially safer vectors has allowed the genetic modification of a higher number of HSPCs leading to the initiation of a clinical trial for β -thalassaemia²³ and a clinical trial for the lysosomal storage disorder adrenoleukodystrophy²⁴. The latter trial was based on the rationale of over-expressing the functional lysosomal enzyme in HSPC-derived tissue macrophages and microglia. In both trials some clinical benefits have been observed: in the first trial one adult patient had a stable improvement in haemoglobin levels, in the second trial progression of the disease was halted in treated children. Successful results have also been reported for haemophilia B in a recent clinical trial (see 1.3.4.3)²⁵. Other successful results have been recently reported also for Duchenne muscular dystrophy, Parkinson disease and for cancer immunotherapy (through the genetic modifications of T cells)⁵.

As we have now reached a more balanced view of the risks and benefits of gene therapy, the unavoidability of adverse effects has been gradually accepted as the price we pay for otherwise effective therapies. Retrospectively it was unrealistic to ask of gene therapy what no other therapy has ever provided in medicine, efficacy without side effects. Gene therapy for SCID continues to show promise, with more than 90 patients treated, with the vast majority experiencing clinical benefit and the overall 5% mortality being much lower than the 25% mortality risk in allogeneic bone marrow transplantation, which is the current standard of care²⁶. Pharmaceutical companies are now filing the first applications to the institutional drug agencies for marketing gene therapies.

Whereas the therapeutic potential of gene therapy is becoming more established, the risk-benefit ratio needs to be carefully evaluated in each condition. The recurrence of insertional mutagenesis and other immune-mediated side effects calls for continued vector improvements, careful pre-clinical risk assessment and molecular and immunological follow up of treated patients. The “bench to bedside and back” cycle between clinical and laboratory animal investigations is crucial to drive these complex biological therapies forward. The continuous improvements and sophistication of genetic engineering tools can be coupled with the growing capacity of isolation and manipulation of stem cells, including reprogramming and differentiation and promise that more safe and effective therapies for currently untreatable diseases are to come in the near future. Thus a productive marriage is envisioned between stem cells and gene therapy²⁷.

Even if the scientific and clinical maturity may be reached, nowadays the large-scale production of these complex biological therapies to Good Manufacturing Practice (GMP)-grade remains a biotechnological challenge and constitute one of the most complex organizational and regulatory areas currently approached by preclinical research⁵. The ultimate success of gene therapy strictly depends on a virtuous cycle between a rich open-minded society, academic scientists, physicians, small biotechnological companies and the large pharmaceutical industry²⁷.

1.1.2 Gene therapy strategies

Gene therapy strategies can be classified in gene replacement, gene addition, gene subtraction and gene correction¹. Gene replacement involves the delivery of the wild type (wt) gene encoding for the protein that is missing or dysfunctional in target cells, owing to genetic mutations and it represents the prototypical protocol devised to treat monogenic diseases. Gene addition consists of the delivery of a gene conferring novel functions normally not present or present at lower levels in target cells. For instance, this can be useful to induce drug resistance or to alter immune responses to counteract infectious diseases or cancer. Gene subtraction can be achieved by down-regulating the expression of endogenous genes, exploiting the RNA interference (RNAi) pathways, or by disrupting the coding sequence of the endogenous gene, exploiting genome editing technologies, such as sequence-specific engineered endonucleases. Gene subtraction

strategies are intended to counteract the effect of gain-of-function mutations or fight infectious diseases, e.g. by targeting directly the genome of a virus or one of its cellular receptors. Gene correction means repairing genetic mutations at their own site or introducing a functional cDNA downstream of the endogenous promoter and can be obtained by exploiting the mechanism of homologous recombination (see 1.1.7). Also, introduction into target cells of a functional cDNA coupled with a strong splice acceptor may favour the splicing of the endogenous mutated transcript with the functional mRNA, thus generating chimeric mRNAs encoding for a wt protein. This procedure is referred to as *trans-splicing* and can be considered a gene correction approach, although this is not performed at the genome level. Although challenging, gene correction is considered the ultimate goal of gene therapy as it would restore the endogenous control of gene expression with minimal impact on the genome. As the technologies to perform genome editing evolve, clinical application are envisaged for the near future²⁷. More recently, the development of antisense oligonucleotides able to interfere with the endogenous splicing machinery has opened the possibility to force endogenous transcripts to avoid the inclusion of the mutated exon (usually containing a premature stop codon) and can generate incomplete but still functional proteins. This procedure is referred to as exon skipping²⁸.

Regardless of the approach, any gene therapy protocol comprises (i) the therapeutic gene (transgene) or the regulatory nucleic acid, (ii) the target cells and (iii) the delivery method (vector). All gene therapy approaches can target only somatic cells, since the genetic modification of germline stem cells and its eventual transmission to the progeny is not allowed for ethical reasons and must be carefully avoided²⁹.

The desired gene therapy strategy can be accomplished by an *in vivo* or *ex vivo* approach. *In vivo* approaches involve the administration of the vector directly to patients, either by localized means (tissue injection) or by systemic means (intravenous injection). *Ex vivo* approaches involve the isolation of stem, progenitor or differentiated cells from patients, their *in vitro* genetic modification and reinfusion into patients²⁷.

1.1.3 Gene transfer vectors

The delivery vectors need to fulfil a series of requirements. They need to be produced in sufficient quantity and purity and formulated in a relatively small volume

that can be administered. In the case of virus-derived vectors, the absence of a replication-competent virus contaminating the vector preparation must be guaranteed. Vectors need to be able to reach the target cells, enter the nucleus, persist and sustain expression of the transgene for the time needed to meet the therapeutic purpose (Figure I). Vectors can mediate integration of their genetic cargo in host cells' chromatin (integrating vectors) or remain in the nucleus as an extrachromosomal element (episome), which is lost as cells cycle, unless able to replicate¹. Recently artificial chromosomes are being developed; they contain the minimal functional elements (centromere, telomeres) and are transmitted at mitosis³⁰. In some cases, such as in gene replacement therapy, lifelong vector persistence and transgene expression is desirable, thus integrating vectors are needed when targeting dividing cells or stable episomes can be used in tissues with relatively slow turnover such as muscle, liver and brain. In other cases, when the aim is to induce a specific temporary response, transient gene transfer or expression is preferable^{1, 31, 32}.

In most cases, vectors cannot incorporate the entire gene, thus the cDNA is used. The absence of maturation of the primary transcripts may result in mRNA instability and inefficient nuclear export, thus splicing sites may need to be re-introduced when possible or compensatory elements have to be included. In addition to the transgene, gene therapy vectors have to carry the elements necessary to express it, such as transcriptional regulatory sequences and a polyadenylation signal (polyA). Together the transgene plus its expression regulatory elements are referred to as expression cassette²⁹.

1.1.4 Transgene expression control

Expression of the transgene can be directed by promoter/enhancer sequences of viral or eukaryotic – including human – origin, complete or partially reconstituted, chimeric or synthetic. Promoter/enhancers can be exogenously regulated, relatively strong or weak, ubiquitously expressed or tissue/cell type-specific. The most appropriate choice is dependent on the diverse therapeutic need. In gene replacement strategies, sustained life-long expression is desirable. Some applications require cell type-specific transgene expression, in other cases robust ubiquitous expression is preferable. In some cases switching transgene expression on and off may be necessary. Depending on whether the

transgene is integrated or not and according to the integration sites, its expression may vary. Transgene expression can also be epigenetically silenced during chromatin remodelling, such as in cell differentiation processes, or by genome surveillance mechanisms³³. In most cases size constraints in vectors ability to incorporate nucleic acids (cloning capacity) limit the number and length of regulatory elements that can be included and often results in imprecise transcriptional control and undesired expression. A recently proposed approach to improve the stringency of transgene expression regulation is to exploit the differential expression of microRNAs between cell lineages or differentiation states (see also 1.4.1)^{34, 35}. By incorporating the target sequence for a microRNA in the 3' untranslated region (UTR) of a transgene, its expression is suppressed specifically in the cells that express that microRNA and expression can be de-targeted from unwanted cell types. Since this control is post-transcriptional, it can be combined with transcriptional control to obtain sophisticated transgene expression patterns³⁶.

1.1.5 Immune response

One of the most significant obstacles against successful gene therapy is the host immune system (reviewed in³⁷). An immune response can be raised against the vector, the transgene or both (Figure I). Most patients (and animal models) affected by a genetic disease are immunologically naïve to the wt gene product and therefore are prone to initiate an immune response against therapeutic transgenes. Gene therapy vectors, especially virus-derived vectors, can be highly immunogenic, which is also true for non-viral vectors (see also 1.3.2). Immune responses can be divided into innate and adaptive, the latter ones comprising both humoral and cell-mediated. Innate responses to vectors can cause local or systemic toxicities immediately following administration and enhance adaptive anti-transgene immunity. Pre-existing humoral responses to vectors can block vectors' ability to enter cells. Most virus-derived vectors induce primary humoral immune responses that would probably limit any strategy that require re-administration of the same vector. Primary humoral and cell-mediated immunity to transgenes can neutralize their protein product or eliminate transgene-containing cells, or both. The development of such immune responses represents a serious risk, since it may result in a failure of the therapeutic strategy and may also expose the patient to the

risk of developing autoimmunity and becoming refractory to other types of replacement therapy. A number of different factors dictate whether an anti-transgene immune response is initiated or not, including the transgene itself, the type and dose of vector, the route of administration, the host genetic background and underlying disease mutation. The accumulated experience indicates that transgene expression in antigen presenting cells (APCs) is one of the major determinants of anti-transgene immunity. Therefore, restricting transgene expression to target cells via tissue/cell type-specific promoters can reduce the probability of such an event (see also 1.4.1)^{34, 38-42}. Over the years, extensive investigations have been performed in order to avoid detrimental immune responses in many gene therapy approaches. Immune responses are instead desired when developing genetic vaccines to fight infectious diseases or cancer gene therapy, as anti-vector immunity may in some cases be helpful in amplifying anti-cancer immunity.

1.1.6 Insertional mutagenesis

When integrating vectors are used, a crucial issue to be faced is insertional mutagenesis. Integration of exogenous DNA into the host genome can provide an initiating mutation that causes expansion of a clonal population which, upon selective pressure and accumulation of secondary mutations, may progress to neoplastic transformation⁴³. Depending on the insertion site and the type of vector, the integrated vector can cause the generation of over-expressed, chimeric (with parts of the vector), truncated or inactive transcripts that can in turn promote cellular transformation.

The main mechanisms of insertional mutagenesis comprise promoter/enhancer insertion and splice sites/polyA insertion. Transcriptional enhancement is probably the most frequent mechanism of gene activation by insertional mutagenesis: integrated vectors could initiate chimeric, spliced transcripts from their own enhancer/promoters and/or provide an enhancer effect on host transcription, thus determining an up-regulation of the genes neighbouring the insertion site, with transcription starting from their physiological transcription start site. Vector-derived enhancers may also affect the expression activity of genes located at a distance because of chromatin loops. Integration in intragenic regions could also happen. In this case, *cis*-acting sequences located in the vector (such as deliberately introduced or cryptic polyA and splice sites)

could affect the result of transcription, leading to the synthesis of aberrant mRNAs. In most cases this would result in loss-of-function mutants and the other allele would still exert its normal function, however truncated or aberrant proteins may possess dominant negative properties or display enhanced or reduced activity and induce tumourigenesis. Enhancer activation could act in concert with these vector-induced premature-termination/aberrant-splicing events, thus generating high levels of a mutant protein⁴⁴. Over the years insertional mutagenesis has represented a significant burden in gene therapy and a flurry of studies have investigated the causes of these events, devised ways to decrease them and developed tools and models to predict their occurrence and it is still an active area of investigation²⁷.

1.1.7 Site-specific integration

Site-specific integration is considered the holy grail of gene replacement therapy since it would ensure predictable and stable transgene expression with minimal consequences to cellular genomic regulation. Site-specific integration of template DNA can be exploited for gene correction strategies, for example, repairing genetic mutations at their own site or introducing a functional cDNA downstream the endogenous promoter⁴⁵. Alternatively site-specific integration can target the insertion of an autonomous expression cassette into a specific genomic locus that is permissive and tolerant to integration and transgene expression, so called “safe harbour”⁴⁶.

Site-specific integration can be achieved by providing target cells with a template DNA carrying sequences homologous to the desired site and exploiting the cellular machinery of homologous recombination to insert the exogenous DNA in that specific site⁴⁷. This process has been exploited to perform gene targeting, typically in mouse embryonic stem cells, to obtain the knock out (KO) of endogenous genes, or knock-in of DNA elements or specific mutations and generate genetically modified animals. In order to direct homologous recombination at specific sites and improve the efficiency of the process, engineered sequence-specific endonucleases have been developed, such as zinc finger nucleases (ZFNs)⁴⁸. The availability of these designer sequence-specific nucleases has brought the possibility to perform targeted genome modification within the reach of gene therapy. Upon such nucleases generate a site-specific double-strand break in the genome, target cells can repair it by the error-prone mechanism non-

homologous end joining, which can be exploited to perform gene disruption. Alternatively, the break can be repaired by homologous recombination of homologous template DNA co-delivered with the nucleases to target cells, giving rise to the desired modification.

Because efficient site-specific targeting may require expansion and characterization of targeted-cell clones, it is mostly limited to *ex vivo* gene therapy strategies. Recently proof-of-principle of targeted integration has been achieved in the mouse liver by an *in vivo* gene therapy approach (see 1.3.4.3)⁴⁹.

1.1.8 Liver gene therapy

Although *ex vivo* gene replacement strategies using HSPCs are in a more advanced stage of clinical development, the liver remains a highly desired target organ for gene therapy⁵⁰.

The liver is a biochemical factory and hepatocytes carry out most catabolic and anabolic pathways involving biological molecules. Therefore, numerous diseases such as metabolic disorders, lysosomal storage disorders and plasma protein deficiencies have their origin in this organ. Liver-directed gene therapy holds promise not only for the treatment of genetic diseases directly affecting the liver, but also for all other diseases that cause extra-hepatic manifestations, owing to alterations of specific hepatocyte functions. Indeed, expression of the therapeutic protein in hepatocytes and its secretion into the bloodstream could provide benefit to these conditions. Thanks to its high synthesis and secretory activity, the liver can be exploited as a protein factory for other gene replacement or gene addition purposes. Acquired liver diseases such as viral hepatitis, liver cancer and liver failure can also benefit from gene therapy approaches. Liver gene transfer can be performed by *in vivo* or *ex vivo* strategies. *In vivo* strategies are relatively easy as hepatocytes are readily accessible via the blood stream. *Ex vivo* strategies are currently limited by the difficulties of *in vitro* manipulation of hepatocytes and their inefficient repopulation of the recipient liver, particularly in the absence of a selective advantage⁵¹⁻⁵³.

The liver offers unique features for gene therapy. It is the largest organ in the body and is highly vascularised, by a dual vascular supply. The endothelium of hepatic capillaries (sinusoids) has fenestrations that allow macromolecules and viral particles to

reach hepatocytes. Hepatic blood flow accounts for one-fifth of the cardiac output. Hepatocytes have a very slow turnover (<1% under normal conditions) but retain the capacity to proliferate and regenerate the entire liver following some types of injuries⁵³. Another peculiar advantage offered by gene transfer to hepatocytes over other cell types is the likelihood of inducing immunological tolerance rather than immunity to the transgene product. This outcome allows for long-term expression of the therapeutic protein and ensures that the host will be protected in the case of future immunisation with the therapeutic protein. Moreover, since hepatic gene transfer can favour the induction of tolerance it may also be considered for prevention or treatment of autoimmune diseases⁵⁴.

1.1.8.1 Immune tolerance induction upon liver gene transfer

Considerable evidence supporting the tolerogenic properties of the liver has been observed. Among the first experiments was the successful transplantation of a major histocompatibility complex (MHC)-mismatched liver in a pig⁵⁵ and the induction of immune tolerance towards portal vein-delivered antigens⁵⁶. The liver is susceptible to hepatotropic pathogens (such as hepatitis B and C viruses) that can succeed in establishing chronic infection, avoiding eradication by the host immune system. Physiologically, the liver receives blood draining from the intestine and spleen through the portal vein. Thus harmless food antigens, microbial antigens from the gut flora and products of hepatocyte metabolism reach the liver. However, potentially harmful pathogens invading the circulation may also gain access to the liver. While it is advantageous for the organism to maintain tolerance towards harmless antigens, a lack of response against pathogens would be detrimental. As such, the liver needs to maintain a state of hyporesponsiveness to inflammatory stimuli under normal circumstances. Nevertheless the hepatic environment must also be able to mount an appropriate immune response when necessary.

The exact mechanisms by which the liver can skew the immune system towards tolerance and switch between immune tolerance and immune response to antigens remain incompletely understood. Several studies investigating these aspects suggest that the liver follows peculiar immunological rules and that most of its cells are in a broad sense APCs. These APCs generally present antigen in a tolerogenic way (reviewed

in⁵⁷). Conventional and plasmacytoid dendritic cells (DCs), Kupffer cells (KCs), a specialised population of tissue macrophages, as well as liver sinusoidal endothelial cells (LSECs) can present circulating as well as hepatocyte-derived antigens and have tolerogenic properties under steady-state conditions. Nonetheless, these cells express various pattern recognition receptors, such as Toll-like receptors (TLRs) that can sense pathogen-associated molecular patterns (PAMPs) and allow them to initiate innate immune responses. Although these sentinel cells can be stimulated by PAMPs to release pro-inflammatory mediators, the concomitant expression of regulatory mediators (such as interleukin 10, IL-10, or transforming growth factor- β , TGF- β) leads to a state of hypo- or non-reactivity to subsequent stimuli. This hyporesponsive state is achieved through negative regulators of TLR signalling (cell-intrinsic mechanism) or by high local concentrations of immune regulatory molecules. Liver APCs are less prone to induce adaptive immunity upon PAMPs stimulation, whereas similar stimuli would promote strong immunity in other organs.

Liver DCs express low levels of MHC class II (MHC-II) and co-stimulatory molecules and they are poor stimulators of naïve T cells, while promoting the induction of regulatory T cells (Tregs), a well known class of T lymphocytes capable of suppressing effector responses⁵⁸. Unstimulated plasmacytoid DCs (pDCs) have been shown to induce T cell tolerance⁵⁹. KCs are the major producers of IL-10 and TGF- β under steady-state conditions. They can inhibit DC-induced T cell activation and stimulate the suppressive activity of Tregs. These KCs functions can be overridden by strong TLRs activators⁶⁰⁻⁶². LSECs can scavenge and sample circulating and hepatocyte-derived antigens for processing and presentation in MHC class I (MHC-I) and MHC-II molecules. LSECs have also been implicated in promoting tolerance, as T cells priming by these cells fails to induce effector functions, due to low expression of MHC-II, co-stimulatory molecules and pro-inflammatory cytokines⁶³. Tolerogenic programming of T cells by LSECs can be subverted by increasing co-stimulatory signalling. Also hepatocytes have been shown to possess antigen presenting capacity. Lymphocytes circulating in hepatic sinusoids can extend cytoplasmic protrusions entering through sinusoidal fenestrations into the space of Disse where they can contact with hepatocytes⁶⁴. In contrast to other parenchymal cells in non-lymphoid organs in the body, hepatocytes can directly prime T cells⁶⁵⁻⁶⁷. Hepatocyte-primed T cells undergo

initial clonal expansion but are eventually eliminated because of the lack of sufficient co-stimulation and the subsequent activation of pro-apoptotic programs.

While antigen presentation in the tolerogenic liver environment favours the induction of tolerance, the initiation of immune responses can occur after APCs activation and migration to lymphnodes where antigen presentation occur in a canonical context. Thus the outcome of the immune response against an antigen could be the result of the balance between intra-hepatic and intra-nodal antigen presentation. The site of primary T cell activation may thus determine the balance between immunity and tolerance. T cell activation with subsequent tolerance may prevail when intra-hepatic antigen presentation is greater or begins earlier than intra-nodal antigen presentation⁶⁸.

Potentially, the inherent tolerogenicity of the liver could be exploited to prevent or suppress unwanted immune responses. Targeted expression of foreign or auto-antigens in hepatocytes through gene transfer has been shown to induce antigen-specific Tregs and tolerance towards a number of model or therapeutic antigens and using a variety of delivery vectors⁶⁹⁻⁷⁶. Specific tolerance to transgene products can thus be achieved upon hepatic gene transfer, when transgene expression is specifically targeted to hepatocytes and minimised in APCs and the gene transfer vectors cause little innate immunity stimulation and tissue damage. It is unclear whether this outcome can be observed for all types of transgenes or conditions. It is likely that certain transgenes are more immunogenic than others and the host genetic background or the underlying disease may render the host more prone to develop an immune response. In these cases, other immune modulatory approaches can be coupled to liver gene transfer to favour tolerance induction⁵⁴. The growing knowledge of the liver immunoregulatory circuits and of ways to manipulate them may allow the development of powerful approaches for immune-mediated diseases.

1.2 Haemophilia

Haemophilia is an inherited coagulation disorder caused by the deficiency or dysfunction of coagulation Factor VIII (FVIII) or coagulation Factor IX (FIX), the former being referred to as haemophilia A or classic haemophilia, the latter as haemophilia B or Christmas disease (named after the family in which the factor was described). Factor XI deficiency (originally called haemophilia C) is less common and

in most cases mild coagulation disorder. Haemophilia has an X-linked recessive inheritance and is due to mutations in FVIII-encoding (*F8*) or FIX-encoding (*F9*) gene. Haemophilia has a prevalence of 1 in 10,000 males worldwide (1 in 5,000 for haemophilia A, 1 in 30,000 for haemophilia B). Male individuals are clinically affected, while women are carriers and are generally asymptomatic⁷⁷.

Haemophilia is notorious from a historical perspective since Queen Victoria of England was a carrier and the trait was passed along the royal families of Prussia, Spain and Russia during the XIX and XX centuries. Recent molecular analysis of original specimens from the Romanovs revealed mutations in the *F9* gene, thus the royal disease turned out to be the less common haemophilia B⁷⁸.

The *F8* and *F9* genes were cloned in the 1980s and are both located on the long arm of chromosome X. *F8* gene is very large (186 kb, with approximately 9 kb of exons), while *F9* gene measures 33 kb, with approximately 2 kb of exons. Some thousands of different mutations are recorded in databases, with approximately one third of them arising *de novo*⁷⁹. Several types of alterations can affect *F8* gene, including gene rearrangements, missense and nonsense mutations, abnormal splicing, deletions or insertions of genetic elements. One of the most common mutations (affecting about 45% of individuals with haemophilia A) is intron 22 inversion, a unique combined gene inversion that disrupts the gene. During meiosis, crossing over occurs between homologous sequences located nested in the intron and extragenic. This mutation arises almost exclusively in the male germline⁸⁰. Mutations in the *F9* gene have been described in all regions, the majority being point mutations, while short additions or deletion, gross deletions or complex rearrangements are less frequent⁷⁹. Mutations in the promoter have been described leading to the haemophilia B Leiden phenotype, in which FIX levels gradually rise following puberty⁸¹.

FVIII is a 2351-amino acids long glycoprotein that consists of two chains. It can be synthesised and released into the bloodstream by hepatocytes, although the primary site of production in humans is debated and extra-hepatic FVIII production has been reported in several tissues (spleen, lymph nodes, pancreas, kidney, muscle)⁸². Clinical evidence from haemophilia A donor liver transplantation and studies conducted in animal models suggest that FVIII can also be synthesised by other cells, such as endothelial cells and bone marrow-derived monocytes and tissue macrophages and

mesenchymal stromal cells⁸³⁻⁸⁵. The protein contains a large B domain that is not essential for coagulant activity. In the blood, FVIII mainly circulates in a stable non-covalent complex with von Willebrand factor (VWF), an endothelial-cell derived plasma glycoprotein that protects it from proteolysis. FVIII has a half-life of about 12 hours and is found at 100 ng/ml in normal plasma⁸⁶.

FIX is a 415-amino acids serine protease produced by hepatocytes. Because it is less complex than FVIII, FIX has been studied in greater detail. The protein consists of a signal peptide that targets the protein for secretion to the circulation. The propeptide is necessary for posttranslational modification of glutamic acid residues by an intracellular vitamin K-dependent carboxylase. The γ -carboxyglutamic acid residues are necessary for calcium-dependent lipid binding and are crucial to biological activity. Besides γ -carboxylation, the protein undergoes other post-translational modifications, such as disulfide bond formation, beta-hydroxylation and glycosylation. The propeptide is cleaved from the mature protein on transit from the Golgi apparatus before it enters the circulation. During clotting, the activation peptide is cleaved from the zymogen form, resulting in the two-chain active enzyme. FIX has a half-life of about 24 hours and its concentration in normal plasma is 5 μ g/ml⁷⁷.

1.2.1 Pathogenesis

The hallmark of haemophilia is uncontrollable bleeding, either spontaneous or post-traumatic. Bleeding occurs due to failure of secondary haemostasis. During primary haemostasis, formation of the platelet plug is not impaired, but stabilisation of the plug by fibrin is defective, because insufficient amounts of thrombin are generated.

The current model of the coagulation system involves a series of reactions that culminate in the production of thrombin, the key effector enzyme. Each of the reactions relies on the formation of a surface-bound complex and the conversion of inactive precursor enzymes (zymogens) into active proteases. Inactive factors are indicated by roman numerals and the active form by the addition of a lowercase "a". Under normal conditions, natural anticoagulants mechanisms prevail over the procoagulant activity. The coagulation cascade is triggered in response to damage of the endothelium, exposure of extravascular tissues and platelet activation. Blood coagulation is achieved when a stable fibrin clot forms and subsequent vessel repair and wound healing can

occur (as well as rapid fibrinolysis). Fibrinogen is converted into insoluble fibrin by thrombin (and by the concurrently activated Factor XIII). Thrombin is in turn activated from its pro-enzyme prothrombin. The latter reaction is catalyzed by activated Factor X (FXa), in combination with calcium, phospholipids and activated Factor V (FVa), the central reaction of coagulation, referred to as common pathway (Figure II). Activation of FX occurs either by activated Factor VII (FVIIa) and tissue factor (extrinsic pathway) or by activated FIX (FIXa) in a complex with calcium, phospholipids and activated FVIII (FVIIIa), an essential cofactor. FVIIIa and FIXa associate on the surface of activated platelets, forming the FX-activating complex ("tenase" or "Xase"). FIX activation is catalyzed by activated Factor XI, which is in turn activated by activated Factor XII (intrinsic pathway). Although the classic view of coagulation proposes two separate pathways, FVIII and FIX are known to be central to the process. Activation of FIX indeed occurs as well by the tissue factor-VIIa complex. Once coagulation is initiated, the tissue factor pathway inhibitors (TFPI) block the extrinsic pathway and elements of the intrinsic pathway, particularly FVIII and FIX become the dominant regulators of thrombin generation (Figure II). Haemophilia results from reduced tenase activity, owing to decreased amounts of and/or functionally abnormal FVIII or FIX⁸⁷.

The laboratory tests employed to assay blood coagulation defects include: activated partial thromboplastin time (aPTT), the prothrombin time (PT) and the thrombin time (TT). These tests measure the time of plasma to clot upon addition of calcium and a phospholipidic activator. The aPTT assesses the function of the intrinsic pathway, while the PT assesses the function of the extrinsic pathway, as an excess of tissue factor is added. The TT assesses fibrin formation as thrombin is added in the test (Figure II). The bleeding time instead can detect platelet dysfunction and defects in primary haemostasis. In haemophilia the aPTT is prolonged, while PT and TT are generally in the normal range.

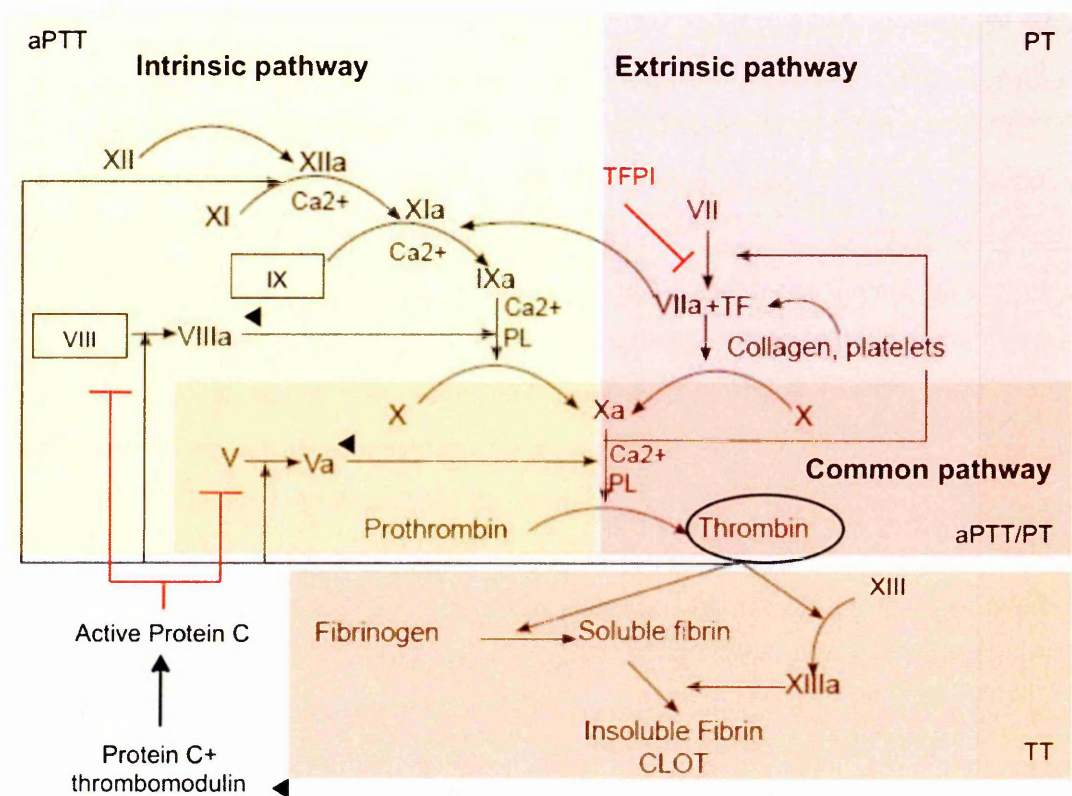


Figure II: Coagulation cascade and laboratory tests to assess coagulation disorders. Schematic of the reactions involved in blood coagulation in vivo and negative regulators. PL: phospholipids. TFPI: tissue factor pathway inhibitors. aPTT: activated partial thromboplastin time. PT: prothrombin time. TT: thrombin time.

1.2.2 Clinical manifestations

Haemophilia A and B are clinically indistinguishable. The disease is characterized by excessive bleeding into various organs. Soft tissue haematomas and haemarthroses are highly characteristic. Haemophilia has been broadly classified in severe, moderate and mild, based on the severity of clinical manifestations. In severely affected patients (<1% of normal activity) recurrent haemarthroses resulting in chronic haemophilic arthropathy occur by young adulthood and are highly debilitating without treatment. Serious haemorrhages can compromise vital organs, however, episodes are intermittent and, except for intracranial bleeding, death because of haemorrhage is rare. Moderately affected patients (1-5% of normal activity) have occasional spontaneous haemarthroses and usually, but not always, following trauma. Mildly affected patients (6-30%) have

rare spontaneous haemorrhages. The disease may go undiagnosed and discovered only following routine pre-surgery testing⁸⁸.

Bleeding into joints accounts for approximately 75% of bleeding in severely affected patients. The joint usually swells, becomes warm and exhibits limited mobility. Repeated bleedings into the joints eventually cause destruction of articular cartilage, synovial thickening and other reactive changes in the adjacent bone and tissues. The major complication is joint rigidity complicated by muscle atrophy and soft tissue contractures. The tendency for repeated bleeding episodes results in the clinically recognized “target joint”. Haemorrhage into subcutaneous connective tissue or into muscles may occur with or without trauma. As a result, large haematomas may form and progressively enlarge in severely affected patients. Retroperitoneal haematomas can expand through the diaphragm into the chest and neck and compromise the airways. Oropharyngeal haematomas, sometimes as a complication of simple colds may enlarge and lead to airways obstruction. Mucous membrane bleeding is common, often resulting from allergic reactions or trauma and can be associated with local lesions involving the respiratory tract. Intracranial haemorrhage is the most dangerous event and can put life at serious risk. Bleeding into the central nervous system may be spontaneous but usually follows trauma. Haemorrhage into the spinal cord is uncommon but can result in paraplegia⁷⁷.

1.2.3 Treatment

There is currently no cure for haemophilia. The treatment aims at raising missing factor to sufficient plasma levels in order to restrain spontaneous bleedings or arrest haemorrhages following trauma or surgery. Bleeding events can be managed by exogenously replacing the missing factor (replacement therapy).

Whole blood transfusion was shown to treat haemophilia-associated bleedings in the 1800s. The modern treatment started during the 1940s with plasma transfusion. However, plasma-transfused clotting factors were not enough to stop severe bleeding⁸⁹. Subsequent advances in blood fractionation and the discovery of the plasma fraction enriched for clotting factors in the 1960s led to the availability of the first factor concentrates, capable of delivering large amounts of clotting factors in a relatively small volume⁹⁰. The expectancy and quality of life of severe haemophiliacs dramatically

improved thanks to such concentrates and home infusion therapy was introduced in the 1970s. After the advent of factor concentrates, the morbidity and mortality in haemophilia were significantly reduced and the life span of haemophilic patients began to approach that of normal individuals. Unfortunately, this advance was overshadowed by contamination of the factor concentrates with hepatitis B virus, hepatitis C virus (HCV) and, later on, human immunodeficiency virus (HIV). Complication of HIV and HCV is the leading cause of death in the haemophilia population treated before 1985, when viral inactivation steps were introduced in the preparation of plasma-derived products⁹¹. However, this procedure does not guarantee the absence of blood-borne infectious agents, as non-enveloped viruses cannot be completely eliminated and transmission of prion disease has been a concern, though never proved. Cloning of the genes for FVIII and FIX has allowed the production of purified recombinant proteins by genetic engineering technologies. These recombinant clotting factors, both licensed in the 1990s, are generally safe in terms of viral disease transmission⁹¹.

Replacement therapy can be administered either in response to bleeding (on demand) or as a prophylaxis, which aims to maintain the missing clotting factor levels at about 1% or higher in order to prevent spontaneous bleedings, especially into joints. Prophylaxis has gradually become the preferred choice, especially in young patients. Regular intravenous (i.v.) infusions of FVIII (3 times/week) or FIX (2 times/week) prevent joint abnormalities in most patients receiving prophylaxis early in life. Factor levels at 30-50% are sufficient to treat mild bleedings, such as uncomplicated haemarthroses or superficial haematomas, while bleeding into deep muscles or large haematomas require factor levels of 50% or higher. The control of serious bleeding affecting pharyngeal or retroperitoneal spaces or the central nervous system necessitates sustained factor levels of 50-100% for 7-10 days. Prophylactic replacement for surgery is intended to achieve normal factor levels (100%) for a period of 7-10 days. Prophylactic replacement therapy with recombinant factors is considered effective and safe and currently patients undergoing this type of treatment from the young age have a nearly normal life expectancy (about 65 years)⁹¹.

Liver transplantation has been performed in some cases in older haemophilic patients co-affected by chronic hepatitis and resulted in a cure of the disease. However it cannot be proposed as a standard therapy⁹².

1.2.4 Inhibitors development and management

The major complication of factor replacement therapy is the formation of allo-antibodies against the supplied factor that can neutralize its activity. Neutralizing anti-factor antibodies are frequently referred to as inhibitors. Inhibitors develop in 20-30% of patients with severe haemophilia A and 3-5% of patients with haemophilia B following replacement therapy⁹³. Rarely, inhibitors can arise as auto-antibodies against clotting factors leading to acquired haemophilia⁹⁴. Mutations with substantial loss of coding regions, such as large deletions or inversions, predispose patients to inhibitor development and represent the main predictor of this complication, as central immune tolerance may not develop towards the missing clotting factor. Family history and genetic background, such as African descent, some *HLA* haplotypes or the genetic profile of immune regulatory genes are other risk factors. Usually inhibitors appear early after the initiation of replacement therapy. However, the risk of inhibitor formation is increased during intensive replacement therapy, such as for trauma, major bleeding or surgery, as the latter ones can represent “danger signals” for the immune system to attack the supplied factor. On the contrary, the degree of purity of the replaced factor does not seem to affect the probability of inhibitor formation⁹⁵.

The clinical management of inhibitor-positive haemophilic patients is challenging. Inhibitors are diagnosed in an aPTT assay in which normal plasma does not correct the aPTT in a 1:1 mix with test plasma. The Bethesda titre is defined as the inverse of the dilution of test plasma that inhibits 50% of normal plasma (1 Bethesda Unit, BU = 1:1 dilution). Based on the inhibitor titre, patients are arbitrarily classified in low responders (<10 BU) and high responders (>10 BU). Treatment in inhibitor patients aims at controlling bleeding episodes and eradicating the inhibitors. In low responders, bleedings can be managed by administering high doses of recombinant factor which can overcome inhibition, or recombinant factor from other species such as porcine FVIII, if inhibitors do not cross-react. High-responder patients usually do not respond to FVIII or FIX concentrates and the control of bleeding events can be achieved using bypassing agents, such as prothrombin complex concentrates (containing variable amounts of activated factors such as FVIIa, FIXa, FXa) or recombinant activated FVII. How these agents bypass the inhibitors is not known, but it is postulated that they enhance the

extrinsic coagulation pathway. Although it is believed that they act at the site of vessel injury, where activated platelets are localized, there is the risk that these agents are thrombogenic^{96, 97}.

The most effective approach for eradicating inhibitors is immune tolerance induction (ITI). ITI is based on the daily administration of high doses of recombinant factor until the inhibitors disappear, which typically requires more than one year. Recently low-dose regimen have been shown to be as effective as high-dose regimen and thus are being preferred in the clinical practice. ITI can be combined with immune suppression or immune adsorption protocols. ITI has a success rate in the range of 60%, but is very expensive and demanding. Complications due to central venous access may occur. Because of the lower frequency of inhibitor development, there is less experience in the management of inhibitor patients with haemophilia B. ITI can be attempted in these patients, but the risk of anaphylaxis or nephrotic syndrome is higher^{98, 99}. The mechanism by which ITI acts is not completely understood. It has been hypothesized that chronic exposure to the antigen in “non-dangerous” conditions (without activation of innate immunity) induces immune tolerance. Induction of Tregs and anergy/inactivation of memory B and T cells have been reported¹⁰⁰. The management of patients with inhibitors resistant to eradication is very challenging: classic immune suppression or administration of monoclonal anti-CD20 antibodies are generally ineffective. Inhibitors increase both morbidity and mortality in haemophilia and in general the management of inhibitor-positive haemophilic patients is still an unmet medical need.

1.2.5 Bioengineered clotting factors

Recently a number of novel approaches have been conceived to bypass or improve current factor replacement therapy regimens. In particular, increasing the circulation half-life of clotting factors is desirable, as it would reduce the frequency of infusions needed to maintain a mean trough of 1% above baseline, reducing patient inconvenience and non-compliance to therapy. Towards this goal, several bioengineered versions of the clotting factors have been developed¹⁰¹. Early attempts to improve the stability of recombinant clotting factors by conjugation with high molecular weight molecules such as polyethylene glycole (PEGylation) have been mostly unsuccessful¹⁰¹. Another

approach consists in the fusion of recombinant FVIII or FIX to the fragment crystallisable (Fc) domain of human immunoglobulin (Ig). The Fc domain permits binding to recycling receptors that rescue antibodies and Fc-fusion proteins from lysosomal degradation upon phagocytosis. Both FVIII-Fc and FIX-Fc have been developed and resulted in improved pharmacokinetic profile in clinical trials, with 1.5- to 3-fold increased half-life^{102, 103}. Interestingly the more modest increase observed for FVIII-Fc suggest a predominant role for VWF in determining FVIII half-life. Alternatively FIX has been fused with albumin, showing promising result in a clinical trial¹⁰⁴. Other approaches to enhance thrombin formation bypassing the intrinsic pathway have been tried. These include inhibition of key negative regulatory pathways such as TFPI or activated protein C inhibition, or the use of factor muteins such as FVIII-independent FIX or FVIII/FIX-independent FX^{101, 105}. Although promising, the efficacy and safety (in terms of immunogenicity or thrombogenicity) of these approaches need to be determined with more extensive investigations.

1.3 Haemophilia gene therapy

Prophylactic replacement therapy with recombinant products, which are devoid of contaminating viral pathogens, is the current standard of care for haemophilia in high-income countries (see 1.2.3). This form of treatment is considered effective and safe and has substantially improved the quality and expectancy of life of severe haemophilic patients. Nevertheless this treatment has several drawbacks. First of all, the high cost of recombinant clotting factors results in a considerable expense for the health care system (estimated about 100,000 \$ *per year per patient*). Second, the short half-life of recombinant clotting factors imposes frequent (2-3 times *per week*) i.v. injections to minimise the risk of spontaneous bleeds, with resulting discomfort and inconvenience for patients and in some cases non-compliance (central venous access devices may be necessary with the related risks of infections or thrombosis). Third, factor replacement therapy entails the serious risk of inhibitors formation, which challenges further treatments (see 1.2.4). It should be underlined that today about 80% of haemophiliacs live with any or unsatisfactory treatment world-wide, mainly in developing countries¹⁰⁶. For these reasons the development of alternative forms of treatment is encouraged.

In contrast to replacement therapy, gene therapy offers a greater prospect of a definitive cure for haemophilia, through restoration of endogenous, life-long production of the missing clotting factor at therapeutic levels. Haemophilia is an ideal target disease for gene therapy for a number of important reasons. The disease-causing genes are known and have been cloned. The therapeutic goal is relatively modest, as clinical experience indicates that stably rising FVIII or FIX levels to just as 1% of normal levels can significantly ameliorate the bleeding diathesis. These levels do not require tight regulation, since up to 150% of normal are considered safe¹⁰⁷. Although the liver is the natural site of production of most plasma protein, FVIII and FIX can be expressed in a variety of cell types, as soon as biologically active molecules can reach the circulation. The outcome measures are quite straightforward as factor levels and clotting activity can be assessed relatively easily with a blood withdrawal and these levels correlate well with the clinical phenotype. Finally excellent animal models that closely recapitulate the human disease are available for pre-clinical testing of gene therapy strategies. Both KO mice for *F8* and *F9* genes have been generated and dogs affected by haemophilia A and B due to spontaneous mutations are being maintained¹⁰⁸. While haemophilia can benefit from gene therapy, the vice versa is also true. The development of gene therapy strategies for haemophilia, where the therapeutic goal is relatively easy to accomplish, has served as trailblazer for applications in more demanding monogenic and complex diseases. The aforementioned features of haemophilia and the significant limitations of its current treatment modality justify more than 20 years of intense research in haemophilia gene therapy. A variety of gene delivery systems adopting *in vivo* or *ex vivo* approaches and targeting different tissues (most often liver and muscle) have been reported with varying degree of success in preclinical small and large animal models and clinical trials^{107, 109-113}.

1.3.1 Ex vivo gene transfer

Ex vivo gene therapy strategies for haemophilia are attractive because of the hurdles of direct *in vivo* gene delivery. The very first attempts in haemophilia gene therapy were represented by *ex vivo* strategies, but were mostly unsuccessful due to low levels of circulating clotting factors and limited survival of genetically modified cells after return into the host¹¹⁴. *Ex vivo* genetic modification of fibroblast with FVIII-expressing vectors

and implantation into immune deficient mice resulted in persistence of viable fibroblasts for 2 months, capable of FVIII secretion in tissue culture. However plasma FVIII expression could not be detected in mice¹¹⁵. The first clinical trial was carried out by *ex vivo* modification of fibroblasts obtained from 2 patients affected by severe haemophilia B with a FIX-expressing vector and re-implanted back into patients. Expression of active FIX in the plasma was observed at 2% of normal levels, however the cumbersome nature of the procedure was prohibitive and prevented the initiation of further trials¹¹⁶. A subsequent trial was held in which fibroblasts from patients affected by severe haemophilia A were transfected *ex vivo* with DNA plasmids encoding hFVIII. However, upon re-implantation of the cells, FVIII expression was transient and no significant clinical benefit was detected¹¹⁷.

Ex vivo strategies are gradually regaining interest along with the advances in the technology of *in vitro* manipulation and genetic modification of human cells. The main difficulty lies in establishing the appropriate environment and blood supply for implanted cells to survive and release the desired clotting factor into the bloodstream. One interesting approach is to target FVIII expression in endothelial cells, which naturally express the protein and its carrier VWF. Transplantation of wt LSECs in haemophilia A mice following damage to recipient LSECs to facilitate engraftment restored normal haemostasis⁸⁴. In a recent study by Kren et al. efficient targeting of the FVIII transgene has been demonstrated to LSECs¹¹⁸. However engraftment of these LSECs still poses a significant hurdle for the success of these approaches. Alternatively circulating blood outgrowth endothelial cells (BOECs) can be isolated from peripheral blood and expanded *in vitro*. Sustained therapeutic hFVIII expression levels could be achieved in immune deficient mice after i.v. administration of transfected BOECs or after subcutaneous administration in scaffolds^{119, 120}. A similar strategy resulted in FIX expression in myoblasts implanted in bioartificial scaffolds engineered to express vascular endothelial growth factor¹²¹. None of these strategies has been tested beyond small animal models and their efficacy and safety assessment requires more extensive investigation.

Due to the large experience with *ex vivo* genetic modification and transplantation of HSPCs, this approach has been considered a valid alternative to *in vivo* gene delivery for treatment of haemophilia¹²²⁻¹²⁴. As expected by the resetting of the immune system

after pre-conditioning and HSPC transplantation, tolerance was achieved to both FVIII and FIX. However, potential toxicities due to ectopic unregulated clotting factor expression in HSPCs and blood lineages remain to be clarified. Recently, novel approaches involving *ex vivo* HSPCs gene therapy have been reported. In one study, HSPCs were gene-engineered with a vector encoding a FVIII cDNA under the control of a megakaryocyte-specific promoter. After bone marrow transplantation, FVIII expression was selectively targeted in platelets and this was sufficient to correct the bleeding diathesis in haemophilia A mice. Interestingly, this happened even in mice with inhibitors against FVIII, likely due to the fact that platelets release FVIII within the forming clot where it may be hardly accessible to neutralizing antibodies¹²⁵⁻¹²⁷. Similarly, FIX could be expressed and stored in platelets granules, normalizing haemostasis in haemophilia B mice¹²⁸. Another approach involves red blood cell-targeted FIX production. Erythroid-derived FIX was shown to be biologically active and to correct the bleeding disorder, following transduction of HSPCs with a vector carrying the human FIX transgene under the control of human β -globin promoter, enhancer and locus control region¹²⁹. A concern for these approaches is whether the production of clotting factors that are normally plasmatic in red blood cells or platelets alters the normal coagulation profile in treated subjects¹³⁰. Moreover, a significant limitation of all approaches involving HSPC gene therapy is the substantial toxicity associated with the transplantation procedure in myeloablative conditions, whereas non-myeloablative conditions may not allow sufficient engraftment or transgene tolerance induction and their clinical relevance requires careful risk/benefit assessment in each specific condition¹³¹.

1.3.2 Non-viral vectors

Non-viral vectors such as simple naked DNA represent the most obvious carrier of a transgene. However it is unlikely that such DNA would survive the journey from the extracellular space to the nucleus of target cells. To facilitate this task, chemical and physical methods have been studied¹³². Plasmid DNA can be delivered in a complex with cationic polymers, liposomes or nanoparticles which improve stability, cell uptake and intracellular trafficking.

The advantages of non-viral vectors are their relative ease and low cost of production, the absence of virus-derived products, the virtually unlimited size of DNA that can be delivered and the potential for repeated administrations. Although it was initially believed that non-viral vectors cause little inflammation and toxicity, bacterial DNA, in particular unmethylated CpG motifs can activate innate immune responses¹. However, the general low efficiency of gene transfer and the transient expression levels are considerable disadvantages of naked DNA vectors for haemophilia gene therapy.

While non-viral vectors can result in good efficiencies of gene transfer in cell cultures, their efficiency for *in vivo* gene delivery is low. One way to achieve relatively efficient hepatocyte transfection (up to 40%) in mice is by hydrodynamic injection¹³³. This technique is based on high-pressure infusion obtained by rapid i.v. injection (3-5 seconds) of DNA in a large volume (10% of body weight). The simplicity of this technique has allowed a wide range of use for *in vivo* studies in mice. However this technique can cause transient liver damage and is not readily suitable for clinical translation, although efforts to render it more feasible and safer have been made in larger animals¹³².

Even with hydrodynamic infusion into the liver, gene delivery can be short lived, unless chromosomal integration or episome stabilization is achieved. Moreover, transgene expression usually decreases rapidly over several weeks, not necessarily because of the loss of vector DNA, but due to the loss of transgene mRNA, for reasons that are not still well understood¹. It has been shown that minicircle DNA vectors can solve this issue. Minicircle DNA vectors are devoid of bacterial plasmid backbone sequences and essentially deliver only the transgene expression cassette. They have been shown to drive transgene expression at levels 2-3 orders of magnitude greater than routine plasmids¹³⁴.

FVIII and FIX transfer has been reported in normal and haemophilic mice using hydrodynamic liver delivery of a DNA plasmid containing hepatocyte-specific expression cassettes optimized for robust expression and stability, by incorporation of locus control regions and matrix attachment regions¹³⁵. Human FIX (hFIX) expression was maintained at therapeutic levels for more than 1 year in haemophilia B mice¹³⁶. While long-term expression of a human B-domain deleted FVIII (hBDD-FVIII; see 1.3.4.2) was obtained in immune deficient mice¹³⁷, a strong humoral anti-transgene

immune response was elicited in haemophilia A mice independently of whether human, canine or murine BDD-FVIII was delivered¹³⁸. Subsequent studies have shown that transient immune suppression or immune modulation with T cell-depleting antibodies, co-stimulatory blockade or Tregs boosters (complex biological molecules known to increase Tregs/T effectors ratio) can promote long-term expression and tolerance to plasmid-mediated FVIII delivery in haemophilia A mice¹³⁹⁻¹⁴¹. These strategies, however, have never been tested in large animal models of the disease and may be difficult to translate in the clinical setting because of the current requirement for hydrodynamic delivery.

1.3.3 Non-viral integration and gene correction strategies

Several methods to mediate transgene integration into the genome of target cells have been developed and may compare favourably with respect to viral vector-mediated integration. Among these, DNA transposons, bacteriophage recombinases and site-specific engineered nucleases have been adopted as alternative strategies for haemophilia gene therapy (see also 1.1.7). These strategies rely on the (desirably transient) co-delivery of the integrase or nuclease proteins together with an appropriate substrate DNA to be integrated¹. *Sleeping Beauty* (SB) is one of the mostly used transposases in gene therapy and can direct non-homologous integration of a transgene flanked by the recognized transposition sites into TA dinucleotides in the genome. SB-mediated integration of a transposon carrying a FIX expression cassette has been achieved in haemophilia B mice through co-delivery via hydrodynamic liver transfection of two plasmids expressing SB and carrying the FIX-expressing transposon¹⁴². The same strategy also resulted in BDD-FVIII expression in haemophilia A mice, although these mice required pre-tolerization to FVIII to avoid inhibitor formation¹⁴³. Recently, a hyperactive SB transposase (SB100X) was derived from the original protein by selection from engineered mutant libraries¹⁴⁴. SB100X could transpose 100-fold more efficiently a FIX-expressing transposon in the liver of normal mice by hydrodynamic DNA delivery, and in haemophilia B dogs through co-delivery of two adenoviral vectors (see 1.3.4.2): one carrying a cFIX expression cassette and the other encoding for SB and a recombinase to circularize the transposon DNA¹⁴⁵. Bacteriophage recombinases, such as ϕ C31, can mediate integration of a DNA sequence

flanked by specific recognition sites into pseudo-sites in the genome that resemble the endogenous prokaryotic consensus sites. Co-delivery of the recombinase and a FIX-expressing DNA by hydrodynamic injection in normal mice resulted in hFIX expression¹⁴⁶. RNA *trans-splicing* has also been obtained *in vivo* in the liver of haemophilia A mice administered with naked DNA containing the trans-splicing construct. This resulted in partial short-term correction of the defective haemostasis¹⁴⁷. Recently, in an elegant study, it was reported proof-of-principle of *in vivo* genome editing in hepatocytes. This study was performed in *ad hoc* generated normal and haemophilia B mice transgenic for a truncated form of human *F9* gene under the transcriptional control of a hepatocyte-specific promoter. Neonatal mice were co-administered with two adeno-associated viral vectors (see 1.3.4.3), one expressing ZFNs designed against the first intron of the *F9* gene and the other carrying a donor DNA bearing a promoterless FIX cDNA spanning exons 2-8. This procedure resulted in targeting of the corrective cDNA to the homologous transgenic sites and expression of FIX at 5% of normal levels⁴⁹. It is unclear whether this approach is feasible in adults, as ZFNs expression would predictably be long-lasting in genetically modified hepatocytes. While powerful and fascinating from the molecular and technological point of view, all these approaches require efficient *in vivo* delivery of the nucleases/template DNA combination and their main limitations fall into the same category as the limitations of the delivery vectors. These approaches may be better suited for *ex vivo* strategies. Moreover, the safety of such nucleases in gene therapy applications has yet to be thoroughly assessed.

1.3.4 Viral vectors

The development of viruses-derived vectors stems from the necessity to find an efficient vehicle to shuttle the foreign genetic materials into the nucleus of mammalian cells^{1, 148}. Recombinant viral vectors are designed to harness the native viral infection pathway to perform this task. Most viral vectors are constructed *ad hoc* to lead to a non-replicative life cycle, which ends with the insertion of the recombinant viral genome into target cells (referred to as transduction). The principle for recombinant viral vector construction is the physical separation of the *cis*- and *trans*-acting genetic elements. This is performed in order to generate a replication-defective viral vector and to

minimize the risk of formation of replication-competent particles. On one side, the *trans*-acting sequences of the original viral genome that encodes for proteins involved in virion assembly and transfer of genetic material are maintained and expressed *in trans* in the producer cells (by packaging constructs). Instead, all the viral genes coding for toxic or pathogenic proteins should be eliminated. On the other side, the engineered vector genome contains the therapeutic transgene expression cassette together with the only *cis*-acting elements from the original virus necessary for gene transfer purposes. This is the only nucleic acid sequence containing the packaging signal, encapsidated into viral vector particles and transferred to target cells. The packaging constructs and recombinant vector genome can be provided by transient plasmid transfection or stably expressed in the producer cells, if available.

Viral vectors can be broadly categorised in integrating and non-integrating, depending on their ability to mediate integration of the vector genome into recipient cells' nuclear DNA. Efficient integration is a property of gammaretroviral and lentiviral vectors, while adenoviral and adeno-associated viral vectors are classified as non-integrating vectors. These are the most widely used viral vectors in gene replacement therapy.

1.3.4.1 Gammaretroviral vectors

Gammaretroviral vectors (RVs) derive from gammaretroviruses, belonging to the family of *Retroviridae*. This family comprises several animal viruses that are now being exploited as gene transfer vehicles: alpharetroviruses, gammaretroviruses, lentiviruses and spumaviruses. Retroviruses are enveloped viruses of about 100 nm with a 7-10 kb positive single-stranded RNA (ssRNA) genome present in two copies inside the particle. For productive infection, the viral genome must be converted into double-stranded DNA (dsDNA) by the viral reverse transcriptase, packaged inside virions. Once the reverse transcribed genome is transferred to the cell nucleus by means of passive or active mechanisms, all retroviruses are able to integrate their genome into the host cell chromatin. Gammaretroviruses harbour a simple genome bearing the essential genes *gag* (encoding for capsid proteins), *pol* (encoding for reverse transcriptase, integrase and protease) and *env* (encoding for the envelop protein). Lentiviruses and spumaviruses have a more complex genome harbouring the essential genes together

with additional regulatory and accessory genes responsible for regulation of viral gene expression, life-cycle and pathogenicity²⁹.

Gammaretroviruses were among the first viruses to be exploited to develop viral vectors for gene therapy, because of the extensive knowledge about these viruses, the absence of natural infections in humans and the possibility to produce them with a non-native envelope (pseudotyping). RVs allow the stable transfer of up to 7 kb of genetic material because they integrate into the host cell genome, thus leading to transgene transmission to the cell progeny upon cell division. Over the years, stable packaging cell lines have been developed, thus facilitating the scaling-up of vector production for clinical translation¹⁴⁹. RVs were the first viral vectors to enter clinical trials and have brought important advances in gene therapy with *ex vivo* transduction of HSPCs (see 1.1.1).

A significant drawback of RVs is that they rely on cell mitosis and disassembly of the nuclear envelope to integrate their genome into the target cell chromatin, therefore they can only transduce actively proliferating cells. For this reason they are better suited for *ex vivo* transduction, rather than direct *in vivo* administration. Liver gene transfer by RVs requires stimulation of hepatocyte replication or neonatal delivery in order to achieve efficient transduction. To encourage hepatocyte replication different methods were introduced including partial hepatectomy and growth factor-induced hepatocyte proliferation. The pioneering work by Kay et al. in the early 1990s resulted in the first long-term somatic gene therapy in a large animal model. Haemophilia B dogs were treated by portal vein infusion of canine FIX (cFIX)-expressing RVs upon partial hepatectomy. The procedure resulted in stable low-level FIX expression (0.1% of normal)¹⁵⁰. Subsequent work focused on neonatal or hepatocyte growth factor (HGF)-potentiated administration of FVIII or FIX in haemophilic mice and dogs. Functional hFVIII expression was first obtained upon neonatal RV delivery in haemophilia A mice, yet in some mice inhibitors developed¹⁵¹. Neonatal delivery of cFIX-expressing RVs also resulted in therapeutic transgene levels in haemophilia B dogs¹⁵². Interestingly neonatal liver gene transfer resulted in immune tolerance to FIX, probably due to the immaturity of the immune system of a neonate, while the same RV administered to adult HGF-treated haemophilia B mice induced anti-cFIX antibodies. RV-mediated neonatal liver gene therapy in haemophilia A dogs resulted in therapeutically relevant

FVIII levels which were maintained for nearly 3 years¹⁵³. A phase I/II clinical trial was performed in adult patients with severe haemophilia A involving multiple peripheral i.v. administrations of RVs expressing hFVIII. Adverse events were not observed but very low and transient amounts of FVIII were detected¹⁵⁴.

The genotoxicity of RVs observed in clinical trials for inherited immune deficiencies¹⁵⁻¹⁷ raises important concerns over further use of these vectors in the clinical setting. Although improved RVs have been generated which are less prone to induce insertional mutagenesis, the genotoxic potential of RVs remains an issue^{1, 27}. This, coupled to the inefficient transduction of quiescent hepatocytes currently restricts their adoption as a possible form of therapy for haemophilia.

1.3.4.2 Adenoviral vectors

Adenoviruses are medium-sized (80-100 nm) non enveloped viruses composed of a nucleocapsid and a linear dsDNA genome. Several different serotypes have been isolated, many causing upper respiratory tract and gastro-intestinal infections in humans. Adenoviruses were also among the first viruses exploited as recombinant viral vectors for gene therapy. Recombinant adenoviral vectors (Ads) present a number of attractive features for gene therapy. Ads are relatively easy to produce at high titres, efficiently transduce a variety of cell types including non-dividing cells, can accommodate large amounts of DNA and efficiently express transgenes episomally without integration into the host cell genome. Ads trigger massive innate and adaptive immune responses which represent a major obstacle for their clinical use in gene replacement therapy¹⁵⁵.

To obtain recombinant replication-defective vectors, first generation of Ads was constructed by deleting from the viral genome of origin the early region (E) 1 and E3 genes, responsible for the expression of early viral genes and replication. First-generation Ads were shown to mediate FIX and FVIII expression in mice and dogs upon i.v. administration. The first evidence of FIX expression with Ads was achieved in normal mice¹⁵⁶. The study was then extended to haemophilia B dogs, in which portal vein administration of first-generation Ads resulted in cFIX expression at supra-physiological levels which declined over a period of 100 days. This decline was consistent with gradual loss of vector DNA from transduced hepatocytes. At that time it

was not clear whether this loss was due to dilution/instability of Ad episome, a cytotoxic immune response or both. Mild hepatocyte necrosis was detected at necropsy and a mild episode of disseminated intravascular coagulation (DIC) was suspected¹⁵⁷. The inherent features of FVIII, such as mRNA instability, inefficient protein processing and secretion and the protein instability in the absence of VWF, initially delayed efforts to incorporate it from vectors¹⁵⁸. The first evidence of FVIII expression *in vivo* was obtained upon i.v. administration of Ads in normal mice¹⁵⁸. A short form of the FVIII transgene was used, in which the B-domain was deleted. Removal of the B-domain was shown to have no effect on protein function or immunogenicity¹⁵⁹ and was subsequently largely adopted in haemophilia A gene therapy studies. High-levels of hBDD-FVIII (300% of normal) were achieved in the first week and slowly decreased to background in few weeks¹⁵⁸. Administration of lower vector doses resulted in longer-lasting FVIII expression in adult normal mice (5 months after vector injection) and a dose-dependent hepatotoxicity was apparent¹⁶⁰. Subsequently a study was performed which showed, for the first time, proof-of-concept haemophilia A gene therapy in dogs. Peripheral vein administration of Ads resulted in circulating hBDD-FVIII levels well above the therapeutic threshold, which lasted for 2 weeks, when high-titre inhibitors developed¹⁶¹. This event highlighted the need to use specie-specific transgene to more appropriately evaluate the immune consequences of gene therapy. A more in-depth analysis of Ads-triggered toxicity was carried out in haemophilia A dogs treated with peripheral vein administration of Ads expressing canine BDD-FVIII (cBDD-FVIII). In these dogs a biphasic liver toxicity was detected. The first phase occurred within a few days after vector injection and was a dose-dependent response to the Ads particles. The second dose-independent phase arose at 7-10 days after vector injection and was consistent with an adaptive immune response that caused cytotoxic T lymphocyte (CTL)-mediated killing of transduced hepatocytes expressing both Ad backbone viral antigens and the transgene product. This immune response also led to the development of inhibitors. Although a hepatocyte-specific promoter was used, this immune response probably occurred following a strong innate immune activation induced by the Ads¹⁶². Despite the E1/E3 deletion, first-generation Ads, though being replication-defective, could still express viral proteins in transduced cells and this was thought to substantially contribute to the observed hepatotoxicity and immune responses.

A significant advance in Ad technology was the development of progressively attenuated Ads with further deletions of the original viral genome until the latest generation of Ads, termed gutless, high-capacity or helper-dependent Ads (HDAds) was introduced. HDAds are devoid of all viral genes, which are provided *in trans* during vector production by a helper virus and can carry up to 35 kb of DNA between the viral inverted terminal repeats (ITRs)¹⁶³. HDAds were shown to provide sustained FIX or FVIII expression in mice, dogs and nonhuman primates (NHPs). I.v. administration of HDAds carrying the full-length hFVIII cDNA under the control of a 12-kb albumin promoter allowed high-level expression slowly decreasing over a period of 1 year in those haemophilia A mice that did not develop antibodies¹⁶⁴. Ehrhardt et al. developed a HDAd bearing a previously optimized hepatocyte-specific cassette¹³⁵ for hFIX or cFIX expression and containing matrix attachment regions and human centromeric repeats as “stuffer” DNA. This HDAd was able to provide therapeutically relevant FIX expression in haemophilia B mice and dogs at non-toxic doses, lasting for only few months in one dog^{165, 166}. More prolonged therapeutic cFIX expression was obtained in older haemophilia B dogs receiving higher doses of HDAds, but transient hepatotoxicity and thrombocytopenia were observed¹⁶⁷. Therapeutic FVIII levels were obtained in haemophilia A mice and dogs treated with i.v. infusion of HDAds expressing human or canine BDD-FVIII under the control of hepatocyte-specific promoters. In immune competent haemophilia A mice anti-transgene cellular and humoral immune responses were elicited and transgene expression was maintained for only 2 weeks in one dog, even if inhibitors did not develop¹⁶⁸. Longer-lasting expression of cBDD-FVIII without anti-transgene immune response was achieved in haemophilia A dogs receiving higher doses of HDAds containing a more potent hepatocyte-specific expression cassette, but transient hepatotoxicity and thrombocytopenia were observed¹⁶⁹. Low-level expression of cBDD-FVIII and partial correction of the clotting defects were reported to persist for more than 1 year in haemophilia A dogs, but transient acute toxicity was also observed¹⁷⁰. Overall HDAds showed an improved toxicity profile and reduced immunogenicity due to the absence of viral gene expression and attained prolonged clotting factors expression in haemophilic animals. However it became clear that acute toxicity was mostly mediated by the Ad capsid and genome *per se* and was rather independent of whether a first-generation or

HDAd was used¹⁷¹. Brunetti-Pierri et al. reported dose-dependent acute toxicity after systemic injection of HDAds into NHPs. Doses of 5×10^{12} or 1×10^{13} vector particles per kilogram of body weight (vp/kg) resulted in 50% or 100% hepatocyte transduction respectively, but were accompanied by a strong acute inflammatory response which was lethal at the higher dose. This response was characterized by a rise in serum liver enzymes and other markers of liver damage, increase in white blood cell counts, elevation of serum interleukin (IL) -6 and markers of DIC¹⁷².

Further efforts were devoted to understanding the dangerous relationships maintained by HDAds and the immune system and to decrease their toxicity and/or increase their efficacy in order to find a clinically applicable therapeutic window in which a vector dose is effective and not toxic. The interactions between Ads and the immune system are multifaceted (reviewed in¹⁵⁵). Systemic administration of Ads leads to their contact with blood cells and blood-borne proteins, all of which play a role in acute toxicity. Migration of infiltrating leukocytes, platelet aggregation and production of proinflammatory cytokines and chemokines occur as a result of activation of the complement system by Ads. Both neutralizing and naturally occurring non-specific cross-reacting antibodies opsonise viral particles and in turn activate macrophages. Ads can stimulate platelet-activating factor, a shock inducer lipid signalling molecule. Ads activate TLRs both through capsid proteins and vector genome, which is also recognized by a TLR-independent nucleic acid-sensing mechanism known as the inflammasome¹⁷³. It is challenging to find simple ways to dampen the innate immune response because of the many factors that contribute to Ad acute toxicity.

A phase I clinical study was initiated using systemic delivery of a HDAd carrying the full length hFVIII cDNA, but the trial was put on hold, after the transient thrombocytopenia and signs of hepatotoxicity in the first patient treated. These results were never reported in a peer-reviewed format¹⁵⁵.

Recently a minimally invasive method was introduced which improved hepatocyte transduction of HDAds in NHPs. A balloon occlusion catheter is percutaneously placed in the inferior vena cava to occlude blood outflow from the hepatic vein and vector is infused to the occluded liver via catheterized hepatic artery^{174, 175}. This procedure (based on a similar principle as hydrodynamic injection) resulted in therapeutically relevant hFIX expression levels at clinically relevant doses (1×10^{11} vp/kg) in NHPs, that

persisted for up to 3 years after injection. However, after the Jesse Geslinger death in the OT trial, who received 6×10^{11} vp/kg of a second-generation E1/E4-deleted Ad (see 1.1.1) and the results of the abovementioned trial, there is reluctance in further clinical testing of Ads for gene replacement therapy applications. Moreover, naturally occurring infections with adenoviruses can lead to the development of antibodies able to neutralize transduction by related Ads after systemic administration. On the other hand, the strong immune stimulatory properties of Ads make them attractive vehicles for vaccination and cancer gene therapy³⁷.

1.3.4.3 Adeno-associated viral vectors

In the last decade, adeno-associated virus (AAV) vectors have arguably become the vectors of choice for *in vivo* gene therapy and currently the most compelling pre-clinical and clinical results in gene therapy for haemophilia have been achieved using these vehicles. AAVs are small, non-enveloped viruses of the family of *Parvoviridae* with a single-stranded DNA genome of 4.7 kb. The genome comprises ITRs at both ends of the DNA strand, and two genes, *rep* and *cap* required for the life cycle and the construction of the viral capsid. After entry, trafficking to the nucleus and uncoating, the genome of the wt viruses must be converted into dsDNA by viral proteins and the cellular machinery and can be retained in circular episomal forms or integrated into the AAVS1 site. For productive replication, these viruses require accessory proteins, derived from co-infection with adenovirus or herpes virus.

There are several advantages for using AAV vectors in gene therapy. AAV vectors derive from non-pathogenic viruses, are weakly immunogenic, do not express viral proteins and can efficiently transduce both dividing and quiescent cells. Various viral serotypes have been isolated from different species, which show different tissue-tropism and transduction properties. Efficient methods for the production of high-titre vector stocks have been developed and capsid stability allows for purification and concentration during vector preparation¹⁷⁶. Most (>99%) of proviral AAV genomes remain episomal in the nucleus of transduced cells, often in the form of concatameric DNA molecules. However integration can occur, preferentially into chromosomal breaks, perhaps explaining the bias for actively transcribed genes¹⁷⁷. One important limitation of AAV vectors is the small size of the genome of the original virus that

imposes a maximum size of 4-5 kb for the encapsidated DNA. Another challenge is the spread of natural AAV infections in the general population, leading to anti-capsid immune responses which can reduce the efficacy of gene therapy with AAV vectors, if administered in the circulation¹⁷⁸.

Preclinical evaluation of this delivery system has primarily been assessed with the FIX transgene because of the limited cloning capacity of AAV vectors. The prototypical AAV vector is derived from adeno-associated virus type 2 serotype (AAV2). The use of AAV vectors for haemophilia gene therapy has been explored either by intramuscular (i.m.) or liver-directed vector administration. The muscle offer advantages as a target organ since it is directly accessible without systemic vector administration and may be better suited for those haemophiliac patients with concurrent liver disease. However, muscle-directed expression may not efficiently release clotting factors into the bloodstream and disfavours the onset of tolerance induction towards transgene antigens, rather it increases the likelihood of triggering an anti-transgene immune response. On the other hand liver-targeted transgene expression allows potentially higher levels of protein expression and secretion and is more tolerogenic (see 1.1.8.1). Stable hFIX expression at therapeutic levels was demonstrated in normal mice upon liver gene transfer with AAV vectors and in immune deficient mice upon i.m. injections of AAV vectors. In normal mice the i.m. procedure resulted in an anti-FIX antibody response^{179, 180}. These studies were then extended to haemophilia B dogs, showing stable cFIX at 1-2% of normal levels for 8-17 months (longest time reported) and partial correction of the clotting defects without toxicity or anti-transgene immune responses using either the muscle- or liver-directed approaches^{181, 182}. Stable expression of hFIX to therapeutic levels (1-25% of normal) was also achieved for up to 1 year in NHPs¹⁸³. Further studies evaluated improved AAV vectors containing a hepatocyte-specific expression cassette and reported therapeutic levels of cFIX (5-12% of normal) and nearly complete phenotypic correction. These levels were stable for over 17 months and could be achieved even in dogs carrying a null mutation in the *F9* gene and thus more prone to develop anti-FIX immune responses^{39, 184}. Of note, a recent report showed stable cFIX expression in these dogs after 8 years, with no evidence of anti-transgene immunity or long-term toxicity¹⁸⁵. Numerous studies suggest that hepatic gene transfer by these AAV vectors can induce transgene-specific Tregs and tolerance, which protect the host

from subsequent immunological challenges (see also 1.1.8.1)^{69, 72, 186}. On the contrary, FIX inhibitors developed following intramuscular AAV vector treatment in inhibitor-prone dogs while sustained expression and partial correction of the haemostatic defect was achieved in one dog only when transient immune suppression was given at the time of vector administration¹⁸⁷. The risk of inhibitor development, even in dogs carrying point mutations, increased with increasing vector doses and correlated most strongly with increased dose per site¹⁸⁸.

These encouraging preclinical studies led to two phase I/II dose-escalating clinical trials employing AAV2 vectors for the treatment of individuals with severe haemophilia B. In one trial, patients (exclusively with missense mutations) were injected i.m. with 2×10^{11} to 2×10^{12} vector genomes (vg)/kg of an AAV vector expressing FIX. The procedure was safe, but plasma levels of FIX did not rise above 1% and limited clinical benefit was observed¹⁸⁹. In another trial, AAV2 vectors containing a hepatocyte-specific expression cassette was targeted to the liver of severe haemophilia B patients via hepatic artery delivery. AAV vector infusion was not associated with acute or chronic toxicities. In the high-dose cohort (2×10^{12} vg/kg), plasma FIX levels of up to 12% of normal titres were detected. However, this increase was followed by a transient rise in serum transaminases levels, beginning at 4 weeks after vector injection and a concomitant loss of FIX expression was observed. Since anti-hFIX humoral or cellular responses were not detected in treated subjects, this clinical picture and further studies suggest that elimination of transduced hepatocytes was due to an AAV capsid-specific CTL response¹⁹⁰.

This event was unanticipated considering results from preclinical animal models where transgene expression is observed to be long-lived. Instead humans are natural hosts for AAVs infection and previous encounters with the virus can give rise to clones of memory CD8⁺ T cells that can be stimulated following secondary encounter with the specific AAV antigens. In the clinical trial, although patients were selected for the absence of neutralizing anti-AAV antibodies, memory T cells specific for AAV capsid antigens were detected and it is believed that they may cause the clearance of FIX-expressing hepatocytes. Moreover epitope mapping revealed that these anti-AAV2 capsid T cells recognize capsid epitopes conserved among different serotypes¹⁹¹. It was shown that primary human hepatocytes *in vitro* transduced with AAV vectors slowly

degrade capsid remnants, that can be presented by MHC-I molecules for long time after transduction, thus leading to CTL recognition and killing of transduced cells¹⁹².

Based on this experience, the clinical protocol has been modified to include a transient immune suppression, in order to avoid the T cell response until capsid antigens are completely degraded by transduced hepatocytes¹¹³. The appropriate immune modulation regimen was chosen after validation in NHPs that it did not cause acute toxicity or interfere with AAV vector transduction or tolerance induction to the therapeutic transgene¹⁹³. Results of this trial have not been reported yet.

Several studies in pre-clinical models have attempted to investigate the mechanisms of the unexpected anti-AAV capsid immune response observed in the AAV2 liver-directed clinical trial and to devise ways to restrain them. However, it was hard to replicate clearance of AAV vector-transduced cells by capsid-specific CTL in mice, even upon CTL adoptive transfer or vaccination by AAV capsid-expressing Ads¹⁹⁴⁻¹⁹⁶. CTL-mediated lysis of AAV vector-transduced hepatocytes could be inhibited *in vitro* by soluble T cell receptors, however this strategy would need to be personalised for each patient¹⁹². In addition, proteasome inhibition has been shown both to increase transduction and decrease capsid antigen presentation by limiting proteasome-mediated cytosolic capsid degradation and loading in MHC-I molecules, a process already known to negatively impact on AAV-mediated transduction efficiency^{197, 198}. Along the same line, site-directed mutagenesis of surface-exposed tyrosine residues allows the capsids to escape proteasome-mediated degradation and antigenic presentation, lacking phosphorylation and subsequent ubiquitination¹⁹⁹. The use of alternative AAV serotypes has been proposed as a means to limit anti-AAV2 immune responses. In particular AAV vectors based on the NHP serotype 8 (which has less frequent pre-existing immunity in humans than AAV2) have been shown to allow 10- to 100-fold higher transduction of liver in mice than observed with other serotypes²⁰⁰. Moreover AAV8 does not bind heparan sulfate proteoglycan receptor, resulting in decreased uptake by APCs and improved tolerance induction^{201, 202}. Libraries of randomly shuffled capsid from native serotypes can be generated and placed under selective pressure for the desired properties²⁰³. However, evasion from anti-AAV capsid immunity is not guaranteed, as capsid-specific T cells were activated in a recent clinical trial involving intramuscular administration of alternate serotype AAV1 vectors²⁰⁴. One key advance in AAV vector

technology has been represented by the development of self-complementary AAV (scAAV) vectors. The self-complementary design removes the need for host cell-mediated second strand synthesis, the rate-limiting step for AAV transduction, and results in more efficient transduction and transgene expression²⁰⁵.

The combination of some of the highlighted improvements to circumvent the anti-AAV capsid immune response resulted in the generation of novel AAV vectors, which recently reached the stage of clinical testing. These AAV vectors are based on a self-complementary configuration and the more hepato-tropic serotype 8. They carry a novel expression cassette with a minimal hepatocyte-specific promoter and a codon-optimised hFIX transgene. These scAAV8 allowed safe and efficient transduction of the liver in mice and NHP and resulted in stable hFIX expression at therapeutic levels, even after peripheral vein administration^{206, 207}. These results paved the way for a new phase I/II clinical trial for haemophilia B, in which patients suffering from severe haemophilia B have been treated with a single peripheral vein administration of the described scAAV8. Based on the evidence that the anti-AAV capsid immune response was dose-dependent, the rationale behind this new clinical trial was that the use of more efficient vectors would allow therapeutic transgene expression levels at doses that may not re-activate the memory T cells. The three main modifications with respect to the previous trial by Manno et al. (self-complementary design, alternate serotype 8, codon-optimised transgene) were indeed designed to achieve this goal. The results of this clinical trial showed long-term therapeutic hFIX expression in the range of 1-3% of normal levels in 4 patients treated at the first two dose cohorts. At the highest dose (2×10^{12} vg/Kg), 2 more patients initially demonstrated higher hFIX levels in the circulation, but a rise in serum liver enzymes was apparent 8-9 weeks after vector injection and a drop in hFIX levels was detected. Based on the experience of the previous liver-directed trial, the two subjects were immediately started on a course (4-8-weeks) of glucocorticoid therapy and stabilised at about 3-7% of normal hFIX levels for over 1 year. In both subjects AAV8 capsid-specific T cells were detected. Four of the six treated subjects were able to discontinue prophylactic FIX replacement therapy and in the other two the frequency of injections was decreased²⁵. These results showed, for the first time, that gene therapy can provide stable therapeutic hFIX expression levels in humans after a single vector administration. Extension of this study to a larger number of patients will be essential to

better understand the frequency of the anti-capsid immune response occurrence and whether immune suppression by transient glucocorticoid administration will reliably control this response.

Besides the success of the liver-directed approaches, the interest in i.m. AAV vector administration has not subsided. The AAV2 muscle-directed trial clearly demonstrated that stable gene transfer in muscle fibres is feasible, provided that the vector dose *per* site is limited and only patients with a missense mutation are enrolled. It has been shown persistent transduction in muscle biopsies from treated patients for 10 years²⁰⁸. However, hFIX expression levels were subtherapeutic at the dose tested. Higher dosing appeared impractical owing to the high number of required injections, in addition, studies in the dogs suggested that higher doses might induce inhibitor formation¹⁸⁸. To overcome this limitation, the use of alternative serotype AAV1 vectors have been evaluated and demonstrated 50-fold higher levels of expression but with increased inhibitor formation in mice and dogs²⁰⁹. Regional vascular techniques for the delivery of AAV vectors to more extensive areas of skeletal muscle have also been developed, leading to cFIX expression levels up to 10-fold higher than those obtained with classical i.m. administration in haemophilia B dogs^{210, 211}. These results have not been translated clinically yet and the need to perform transient immune suppression remains a concern.

The length and complexity of the FVIII transgene has hindered the development of haemophilia A gene therapy strategies employing AAV vectors. It is not clear whether muscle-directed gene therapy can be applied to FVIII delivery as well, given its large size, association with VWF and susceptibility to proteases in the extracellular space. Thus efforts have focused on liver gene transfer. The packaging limitations of AAV vectors can be overcome by splitting the FVIII light and heavy chains in two different vectors to be co-administered in the host ("dual vector" approach) or by exploiting the propensity of AAV vectors to form concatamers^{212, 213}. Alternatively, a minimal cassette containing the BDD form of FVIII can be adopted²¹⁴. Both these approaches have resulted in therapeutically relevant levels of cFVIII (2-5% of normal) in haemophilia A dogs for up to 4 years²¹⁴⁻²¹⁶. However, the dose required to achieve therapeutic FVIII activity is higher than those clinically tested and it seems that alternative serotype AAV8 vectors did not reproduce in dogs the same improvements observed in mouse studies^{215, 216}. New FVIII transgene engineered for high expression and secretion are

being developed and may facilitate clinical translation of AAV-mediated haemophilia A gene therapy²¹⁷.

Although most AAV vectors remain episomal in transduced cells and these forms contribute to long-term expression, sequencing of vector-genome junctions from target organs in animals has shown that integration can occur¹⁷⁷. The extent of the long-term risk associated with AAV insertional mutagenesis is still debated. A single report showed that neonatal AAV vectors delivery was associated with a higher incidence of late-onset hepatocellular carcinoma (HCC) in a mouse model of a lysosomal storage disorder²¹⁸. AAV integration analysis showed that 4 HCCs harboured integrations in a complex regulatory region that has been implicated in HCC²¹⁹. In another study, high-dose liver-directed AAV administration in adult mice was followed for 18 months after vector injection. There was no change in the rate of HCC compared to control animals²²⁰. More extensive investigations are warranted to further clarify this aspect.

An alternative treatment strategy for haemophilia is based on the delivery of an engineered FVII transgene that can be directly secreted as activated FVII, as a result of intracellular processing. Although FVII deficiency is very rare, recombinant FVIIa has been introduced as a bypassing agent for inhibitor-bearing haemophilic patients (see 1.2.4). Stable endogenous production of this bioengineered FVIIa may also be devised as “bypassing” strategy for naïve haemophiliac patients. AAV-mediated liver gene transfer of the FVIIa transgene allowed complete and partial correction of haemophilia A and B in mice and dogs, respectively^{221, 222}. This strategy offers the advantage of non immunogenicity of the FVII protein and the risk of thrombotic complications appear low below certain doses²²³.

As a direct result of their good efficiency, remarkably low toxicity and immunogenicity profile, AAVs have rapidly gained acceptance as vehicles for *in vivo* gene therapy. In the previously mentioned landmark study, long-term therapeutic FIX expression has been achieved for the first time in haemophilia B patients upon liver gene therapy with AAV vectors²⁵. This study also restored optimism in the field of haemophilia gene therapy. Nevertheless, pre-existing immunity against AAV capsid is still a concern and will deserve further attention. Moreover it is reasonable to predict that only people resulting negative to neutralizing anti-AAV capsid antibodies may benefit from AAV vectors-mediated gene therapy in the near future (when the vector is

administered in the circulation)²²⁴. The incorporation of large transgenes such as FVIII remains challenging.

1.4 Lentiviral vectors

Lentivirus is a genus of the *Retroviridae* family (see 1.3.4.1). Lentiviruses comprise several animal immunodeficiency viruses, including HIV 1 and 2. Many of these viruses have been exploited to generate gene transfer vectors and the most well studied and advanced in clinical development are HIV-1-derived lentiviral vectors (LVs). LVs are hybrid particles, comprising HIV-1 enzymatic and structural capsid proteins and the envelope proteins derived from a heterologous virus (pseudotype). By changing the pseudotype, it is possible to redirect transduction, either widening or restricting the spectrum of target tissue tropism. Most LVs are pseudotyped with the vesicular stomatitis virus glycoprotein G (VSV-G), which makes the vector pantropic. The VSV-G envelope confers stability to the vector particles, allowing long-term conservation and concentration by ultracentrifugation^{225, 226}.

Cis-acting sequences regulate packaging, reverse transcription, nuclear translocation, provirus integration into recipient cell's genome and are maintained in the recombinant vector genome where the expression cassette is included. *Trans*-acting sequences are present in the packaging construct and complement the vector genome construct in the producer cells, encoding virion structural and enzymatic proteins. For LV construction, at least three different constructs are necessary: an envelope protein-encoding construct, a packaging construct encoding HIV-derived proteins, and the vector genome construct, bearing the transgene expression cassette together with the *cis*-acting sequences²²⁷.

Given the highly pathogenic nature of the parental virus, the risk of emergence of a replication-competent lentivirus (RCL) contaminating vector preparation needs to be carefully addressed. RCL might form upon recombination between the genetic sequences used to construct the vector. Advanced-generation LVs are produced by a combination of at least four different plasmids with minimal sequence homology. All the accessory genes have been removed from the packaging constructs, as well as the *tat* gene, implicated in viral pathogenesis, while the *rev* gene, which allows nuclear export of unspliced viral RNAs, has been placed into a third construct, and autonomously expressed under a heterologous promoter. In this way the expression of

all the necessary packaging proteins is complementation-dependent and possible only in producer cells²²⁸. This split design and the minimal packaging constructs alleviate concerns for the generation of a RCL. The fourth construct encodes for the envelope protein of an unrelated virus, which makes generation of a wt virus impossible and recombination among constructs very unlikely because of lack of homology. Moreover, the use of a minimal set of viral genes to package the vector ensures that even the unlikely RCL could not have the pathogenic features of the parental virus (Figure III).

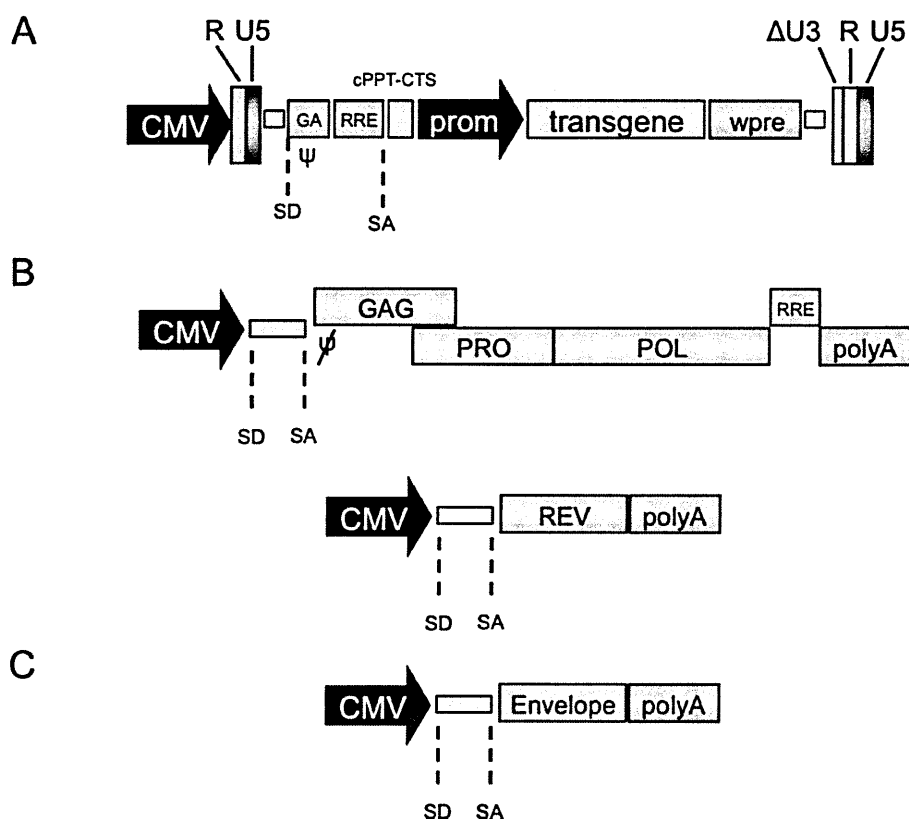


Figure III: Four-plasmid system for the production of third-generation LVs. (A) Transfer vector. The HIV U3 region at the 5' LTR has been replaced by the cytomegalovirus promoter/enhancer (CMV) that allows the synthesis of the full-length RNA for packaging without the need for HIV Tat. A deletion of the HIV enhancer/promoter in the U3 region at the 3' LTR has been introduced. This modified U3 region is copied at the 5' LTR upon reverse transcription in transduced cells, thus obtaining a SIN LV. SD: splicing donor site. SA: splicing acceptor site. ψ : packaging signal, including 5' portion of GAG gene (GA). RRE: Rev responsive element. cPPT-CTS: central polypurine tract to central termination sequence. wpre: woodchuck hepatitis virus post-regulatory element. (B) Packaging constructs expressing the HIV structural proteins and enzymes and the Rev protein. GAG: gene encoding for HIV

capsid, matrix, nucleocapsid proteins. PRO: HIV protease-encoding gene. POL: HIV reverse transcriptase and integrase-encoding gene. polyA: simian virus 40 polyadenylation signal. REV: regulatory viral protein-encoding gene. (C) Envelope construct expressing the envelope glycoprotein(s) from an unrelated virus (pseudotype).

In order to improve the safety of LVs, careful deletions have been made in the viral long terminal repeats (LTRs) to create self-inactivating (SIN) LV²²⁹. This modification abrogates the function of the native viral promoter/enhancer and diminishes the possibility of vector mobilization, as well as LTR influence on genes neighbouring the site of insertion. It also permits the addition of internal promoters, including tissue-specific and regulatable elements, for controlling transgene expression.

Further improvement of the LV vector platform has brought about the reintroduction of the original viral sequence from the central polypurine tract (cPPT) to the central termination site (CTS) at the 5' region of the vector genome in order to increase nuclear import inside transduced cells²³⁰. Vector genome constructs also include sequences like the woodchuck hepatitis B virus post-transcriptional regulatory element (wpre) that, when inserted at the transgene 3' end, stabilizes the transcripts, promoting nuclear export and improved polyadenylation efficiency of both the transgene in transduced cells and the vector genome in producer cells²³¹. The wpre contains a transcriptionally active promoter and the first few amino acids of the woodchuck hepatitis B virus X protein, which has oncogenic potential by still unknown mechanisms²³². Although expression of this small fragment of the X protein has never been demonstrated, precautions have been taken by generating a mutated wpre (wpre*) that lacks the X protein open reading frame and containing mutations in the region putatively crucial for promoter activity²³³. This mutated version (wpre*) has been shown to maintain RNA stabilization capacity and thus has been incorporated into LV constructs which may undertake clinical translation (Figure III).

Importantly, this vector platform has now entered clinical testing in HSPC-mediated *ex vivo* gene therapy, with a reassuring safety profile, both for the absence of detectable RCL and obvious signs of genotoxicity²⁴.

LVs offer several advantages for gene therapy applications. Unlike RVs, they can transduce a variety of cell types, including dividing and non-dividing cells, such as quiescent stem cells, hepatocytes and neurons because LVs exploit active cellular mechanisms for the nuclear import of the viral genome. Integration of the LV into the

host cell chromatin allows stable gene transfer to the target cell and its progeny. LVs can package up to 9 kb of genetic material and transgene expression is unlikely to undergo transcriptional silencing. Importantly, since most of the human population is seronegative, LVs can be injected systemically without the risk of pre-existing immunity against vector components. However, LV technology has limitations as well. For one, vector production still relies on plasmid transient transfection because of the lack of stable packaging cell lines and this represents a hurdle for the industrial scaling-up of vector manufacturing and the production of large quantities of purified LVs. Moreover, considering the integrating nature of LVs, there are safety concerns associated with the risk of germ-line transmission and insertional mutagenesis²³⁴⁻²³⁶.

1.4.1 Liver gene therapy with LVs

LVs are attractive tools for liver-directed gene therapy because of their ability to accommodate relatively large transgenes, stably integrate in non-dividing cells, and lack of pre-existing immunity in most of the human population. Initial studies, using reporter or therapeutic genes, have characterized the biodistribution of LV following intravenous or intraportal delivery to mice. They have shown transduction primarily of the liver, to a lesser extent the spleen and less frequently of bone marrow stroma and lungs^{237, 238}. Early attempts of delivering FVIII and FIX upon systemic administration of LVs resulted in only small percentages of transduced hepatocytes. For this reason it was initially thought that hepatocyte proliferation triggered by partial hepatectomy was needed to obtain efficient transduction by LVs^{239, 240}. The introduction of cPPT and CTS in LV genome increased the transduction efficiency of hepatocytes both *in vitro* and *in vivo* upon intravenous delivery even without partial hepatectomy²³⁷. Systemic injection of mice with LVs expressing hFIX under the transcriptional control of an ubiquitously expressed promoter resulted in up to 4% of normal circulating FIX levels in immune deficient mice^{237, 238}. As expected, it was not possible to obtain the same results in immune competent mice, because a cellular immune response developed against transduced cells and a humoral response developed against the FIX antigen⁴¹.

Further studies were designed to elucidate both innate and adaptive immune responses induced by LV systemic administration. LVs trigger an early and rapidly decreasing inflammatory response which enhances adaptive immunity and limits

hepatocyte transduction. LVs activate pDCs with ssRNA, which is sensed by endosomal TLR7. Upon TLR7 stimulation, pDCs secrete interferon α (IFN- α), which poses hepatocytes in an anti-viral state, reducing their permissiveness to transduction. Type-I IFNs in turn induce the activation of other APCs and transient production of IL-1 and tumour necrosis factor- α (TNF- α)²⁴¹. Moreover, upon intravenous administration, VSV-G-pseudotyped LVs readily transduce and express the transgene in APCs such as liver KCs and spleen APCs, leading to productive antigen presentation to T cells and initiation of an immune response against transduced cells and the transgene products^{237, 242}. Other groups' and our own studies have shown that the introduction of hepatocyte-specific promoters in vectors reduced transgene expression in APCs and subsequent antigen presentation, limiting undesired anti-transgene immune responses. Indeed, liver gene transfer with LVs expressing green fluorescent protein (GFP) or hFIX under the control of hepatocyte-specific promoters circumvented the anti-transgene immune response and resulted in long-term transgene expression in normal mice⁴¹.

However, evaluation of LVs expressing human FIX from hepatocyte-specific promoters failed to achieve sustained FIX expression in haemophilia B mice due to the induction of an anti-FIX cellular immune response⁷³. This occurs because human FIX is a stronger immunogen in haemophilia B mice, which do not express the mouse homolog. Additional analysis suggested that anti-transgene immunity may be a result of off-target transgene expression in hematopoietic-lineage cells of the liver and spleen, because of leaky hepatocyte-specific promoter activity in non-hepatocyte cells and promoter/enhancer trapping events. In order to overcome this problem, the LVs were modified to contain target sequences for the hematopoietic-specific microRNA 142 (miR-142) in the transgene 3' UTR, making it subject to post-transcriptional regulation (see also 1.1.4)³⁴. This novel strategy eliminated off-target expression in hematopoietic-lineage cells, evading the immune response and enabling sustained gene transfer in haemophilia B mice for more than 1 year (longest time assayed) after a single LV administration. Treated mice had around 10% of normal hFIX activity, no detectable anti-FIX antibodies and were tolerant to hFIX, since they were unresponsive to protein immunisation. Importantly, the mice survived tail-clip challenge, thus demonstrating phenotypic correction of their bleeding diathesis⁷³. We have since performed specific immunological studies aimed at shedding light on the mechanism by which this

approach can induce transgene-specific tolerance. We have shown that *in vivo* administration of a miR-142-regulated transgene induces an initial expansion of antigen-specific CD8⁺ T cells which rapidly contract concomitantly with a significant increase of CD4⁺CD25⁺FoxP3⁺ Tregs. These Tregs mediate a robust state of antigen-specific immunological tolerance, which cannot be broken by vaccination and can be transferred *in vivo* to naïve hosts which become unresponsive to antigen-loaded target cells after intravenous administration⁷⁶.

These advanced-design miR-142-regulated LVs which stringently target transgene expression to hepatocytes, by coupling transcriptional and post-transcriptional regulation, hold significant potential for liver-directed gene therapy applications. Their efficacy has been demonstrated in mouse models of haemophilia A and B and in a rat model of hyperbilirubinemia^{73, 243, 244}. Evaluation of these LVs in large animal models is thus crucial towards a potential clinical translation.

1.4.2 Integrase-defective LVs

Integrase-defective LVs (IDLVs) can be generated by packaging the vector with catalytically inactive HIV integrase^{227, 245}. The class I D64V mutation in the integrase catalytic site substantially reduces integration (100- to 1000-fold) without compromising other steps in the transduction pathway²⁴⁶⁻²⁵⁰. Upon transduction, IDLVs can support transgene expression from the non-integrated proviral forms, which accumulate in the nucleus of transduced cells. Because this episomal DNA is progressively lost in actively dividing cells, transgene expression is only transient^{45, 246, 248}. In contrast, IDLVs have been reported to support sustained transgene expression in quiescent mouse tissues, such as the retina and the brain, likely because the episomal vector DNA is retained within the post-mitotic nucleus²⁴⁹⁻²⁵¹.

The ability of IDLVs to achieve therapeutically relevant transgene expression following hepatic gene delivery and to induce immune tolerance have never been studied. We have shown the feasibility of gene replacement therapy and immune tolerance induction using LV-mediated liver gene therapy^{73, 76}. However, concerns remain about the potential long-term adverse effects of vector integration due to insertional mutagenesis. Moreover it is not known whether the tolerogenic outcome of microRNA-regulated LV delivery depends on sustained high levels of transgene

expression within hepatocytes, which requires substantial levels of LV integration and would limit application of this finding outside of gene replacement strategies for the correction of monogenic diseases. These important gaps deserve further studies.

2.AIMS OF THE WORK

The overall objective of this thesis was to gain a more comprehensive understanding of the feasibility, efficacy, safety of liver gene therapy with LVs and its relevance compared to existing technologies, in order to pursue its clinical translation.

The specifics aims of this work were:

1) To evaluate LV-mediated liver-directed gene therapy in a canine model of haemophilia B.

Haemophilic dogs are spontaneous large animal models of the disease and represent a valuable setting to assess the feasibility, efficacy and safety of new strategies aimed at treating haemophilia in patients. Issues such as scale-up of manufacturing, acute toxicity, immunogenicity, efficacy in achieving therapeutic end-points and potentially long-term toxicity can be more stringently and appropriately addressed in dog rather than mouse disease models.

2) To increase the potency (in its pharmacological meaning of efficacy *per dose*) of LVs for liver gene therapy by evaluating improvements in vector and transgene design and in the transduction protocols.

Increasing the potency of gene transfer vectors is crucial in gene therapy applications. It would allow using lower doses to obtain therapeutic benefit, thus reducing potential acute and long-term toxicities and immune activation associated with vector administration, as well as easing manufacturing needs. Towards this goal, we set out to evaluate an alternative envelope, codon-optimised and hyper-functional transgenes and the administration of a proteasome inhibitor before LV, first exploiting the haemophilia B mouse model and then to evaluate the most promising approach(es) in the haemophilia B dog.

3) To evaluate LV-mediated liver-directed gene therapy in haemophilia B mice with pre-existing anti-FIX immunity.

We have already shown that LV-mediated liver gene therapy can induce specific immune tolerance to different transgenes, provided that transgene expression is stringently targeted to hepatocytes. However, the tolerogenic outcome was achieved in mice naïve to the specific antigens. We set out to challenge this outcome by treating mice with induced pre-existing anti-transgene immunity, in order to test the potential of immune tolerance to revert an established immune response. If successful, liver gene therapy may thus become a preferred therapeutic option also for inhibitors-positive haemophilic patients.

4) To evaluate IDLVs for liver gene therapy.

Since insertional mutagenesis is a concern for integrating vectors, we set out to explore the potential advantages of IDLVs to express transgenes in the adult liver, in which hepatocytes turnover is slow. To this aim we assessed the efficiency and stability of IDLV-mediated liver gene transfer as well as the ability to induce immune tolerance, as compared to those achieved with canonical integrating LV.

3. MATERIALS AND METHODS

3.1 Plasmid construction

Plasmid construction was carried out using standard cloning techniques. The plasmids pCCLsin.cPPT.PGK.GFP.wpre, pCCLsin.cPPT.PGK.GFP.wpre.142T, pCCLsin.cPPT.ET.GFP.wpre, pCCLsin.cPPT.ET.GFP.wpre.142T and pCCLsin.cPPT.hFIX.wpre.142T were previously described^{34, 73}. The plasmid pCCLsin.cPPT.ET.OVA.wpre.142T was obtained by exchanging the *NheI*-GFP-*Sall* transgene with the PCR-amplified *NheI*-OVA-*Sall* transgene from pBluescript-OVA (a kind gift from Dr. G. Casorati, San Raffaele Scientific Institute). The plasmid pCCLsin.cPPT.ET.cFIX.wpre*.142T was obtained by first exchanging the *AgeI*-GFP-*Sall* from pCCLsin.cPPT.ET.GFP.wpre.142T with the PCR-amplified *AgeI*-cFIX-*Sall* from pCEP4-cFIX (a kind gift from Dr. T. Nichols, University of North Carolina) and subsequently exchanging *Sall*-wpre-*SacII* with *Sall*-wpre*-*SacII* from pCCLsin.cPPT.PGK.GFP.wpre*. The plasmid pCCLsin.cPPT.co-cFIX.wpre*.142T was obtained by exchanging the wt cFIX transgene (*NheI*-digested and blunt-ended at the 5' – *Sall*-digested at the 3') from pCCLsin.cPPT.ET.cFIX.wpre*.142T with the codon-optimised cFIX *SmaI*-co-cFIX-*Sall* fragment from pBC-co-cFIX (a kind gift from Dr. T. VandenDriessche, Free University of Brussels). The R338L mutation was introduced using QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies), according to manufacturer's instructions. The identity of plasmids was verified by sequencing of the interested pieces.

3.2 Plasmid DNA preparation

Large-scale preparation of plasmid DNA was carried out using the Macherey-Nagel endotoxin-free high purity plasmid maxi prep system, according to manufacturer's instructions. After a modified alkaline/SDS procedure to lyse the cells and precipitate the genomic DNA, the cleared lysate is passed over an ion-exchange column, at the appropriate salt concentration and pH. The plasmid DNA is eluted under high-salt conditions. It is then desalted and concentrated by alcohol precipitation. Plasmid DNA is finally resuspended in TE (10 mM TrisHcl pH 8.0, 1 mM EDTA).

Large-scale preparation of plasmid DNA for large-scale LV production and quality controls were outsourced to Nature Technology Corporation.

3.3 Lab-scale LV production and titration

Third-generation LVs were produced by calcium phosphate transient transfection of 293T cells of the selected transfer vector, the packaging plasmid pMDLg/p.RRE (for integrase-competent LVs) or pMDLg/p.RRED64Vint (for IDLVs), pCMV.REV, the VSV-G envelope plasmid pMD2.G or the gp64 envelope plasmid pBacNPVgp64 and the pAdVantage plasmid (Promega), as previously described²²⁶.

3.3.1 Calcium phosphate-mediated transfection

Nine millions 293T cells are seeded 24 hours before transfection in 15-cm dishes. Two hours before transfection culture medium is replaced with fresh medium. For each dish, a solution containing a mix of the selected transfer plasmid, the packaging plasmids pMDLg/pRRE (or pMDLg/p.RRED64Vint for IDLVs) and pCMV.REV, pMD2.G (or pBacNPVgp64 for gp64-pseudotyped LVs) and the pAdVantage plasmid is prepared using 35, 12.5, 6.25, 9 and 15 µg of plasmid DNA, respectively. A 0.1X TE solution (10 mM Tris-HCl, 1 mM EDTA pH 8.0 in dH₂O) and water (1:2) is added to the DNA mix to 1250 µl of final volume. The solution is left on a spinning wheel for 20-30 minutes, then 125 µl of 2.5 M CaCl₂ are added. Right before transfection, a precipitate is formed by adding 1250 µl of 2X HBS (281 mM NaCl, 100 mM HEPES, 1.5 mM Na₂HPO₄, pH 7.12) while the solution is kept in agitation on a vortex. The precipitate is immediately added to the culture medium and left on cells for 14-16 hours and after that the culture medium is changed. Supernatant is collected 30 hours after medium change and passed through a 0.22 µm filter (Millipore). Filtered supernatant is transferred into sterile 25 x 89 mm poliallomer tubes (Beckman) and centrifuged at 20,000g for 120 min at 20°C (Beckman Optima XL-100K Ultracentrifuge). Vector pellet is dissolved in the appropriate volume of PBS to allow a 500X concentration.

3.3.2 LV titration

For integrase-competent LVs, 10⁵ 293T cells were transduced with serial vector dilutions in the presence of polybrene (16 µg/ml). Genomic DNA (gDNA) was

extracted 14 days after transduction. gDNA is extracted by using Maxwell 16 Cell DNA Purification Kit (Promega) according to manufacturer's instructions. Vector copies *per* diploid genome (vector copy number, VCN) were quantified by quantitative PCR (qPCR) starting from 100 ng of template gDNA using primers (HIV sense: 5'-TACTGACGCTCTCGCACC-3'; HIV antisense: 5'-TCTCGACGCAGGACTCG-3') and a probe (FAM 5'-ATCTCTCTCCTTCTAGCCTC-3') against the primer binding site region of LVs. Endogenous DNA amount was quantified by a primers/probe set against the human telomerase gene (Telo sense: 5'-GGCACACGTGGCTTTTCG-3'; Telo antisense: 5'-GGTGAACCTCGTAAGTTTATGCAA-3'; Telo probe: VIC 5'-TCAGGACGTCGAGTGGACACGGTG-3' TAMRA). Copies per genome were calculated by the formula = [ng LV/ng endogenous DNA] x [n° of LV integrations in the standard curve]. The standard curve was generated by using a CEM cell line stably carrying 4 vector integrants, which were previously determined by Southern blot and FISH analysis. All reactions were carried out in duplicate or triplicate in an ABI Prism 7900HT Realtime PCR thermal cycler (Applied Biosystems). Each qPCR run carries an internal control generated by using a CEM cell line stably carrying 1 vector integrant, which were previously determined by Southern blot and FISH analysis. Titre is expressed as transducing units_{293T} (TU)/ml and calculated using the formula TU/ml = [VCNx10⁵x1/dilution factor]. Vector particles were measured by HIV-1 Gag p24 antigen immunocapture assay (Perkin Elmer) according to manufacturer's instructions. Vector infectivity is calculated as the ratio between titre and particles.

For IDLVs, titre in reverse transcribed units (TU)/ml was determined on 293T cells 3 days after transduction, using an *ad hoc* qPCR, which selectively amplifies the reverse transcribed vector genome (both integrated and non-integrated) discriminating it from plasmid carried over from the transient transfection (RT-LV; ΔU3 sense: 5'-TCACTCCCAACGAAGACAAGATC-3', gag antisense: 5'-GAGTCCTGCGTCGAGAGAG-3'). The amount of human DNA loaded in the reaction was quantified with a qPCR designed to amplify the human telomerase gene as described above.

3.4 Large-scale LV production and quality controls

Large-scale LV production for dog studies were outsourced to MolMed or Genethon.

The vector lots were produced by using a large-scale validated process²⁵². The vectors were produced following pre-GMP guidelines. Briefly, the LV is produced by transient 4 plasmid transfection of 293T cells. 293T cells are derived from HEK 293 cells with stable transfection of the temperature-sensitive SV40 T-antigen; a subclone of the 293T selected for its high yield performance in production of LVs by transient transfection was used for master cell bank establishment; 293T cells from the master cell bank are expanded in 10-tray cell factories and transiently transfected by calcium phosphate precipitation with 4 plasmids as described in 3.3.1. Twenty four hours after removal of the transfection medium, the cell supernatant is harvested and stored at 4°C. The culture medium is replaced and after further 24 hours a second harvest is performed. The medium collected from the two harvests is pooled and filtered through 5/0.45 µm filters to discard cell debris. The downstream purification process includes a benzonase treatment overnight at 4°C, followed by a DiEthylAmino Anion Exchange (DEAE) chromatography step, concentration and gel filtration in PBS or PBS 5% dimethyl sulfoxide (DMSO). The benzonase treatment is aimed at degrading plasmid DNA and producer-cell derived DNA present in the clarified cell supernatant, thus facilitating their removal through subsequent purification steps. Anion exchange chromatography involves the absorption of negatively charged LV particles to positively charged chromatographic support; viral particles are eluted by increasing ionic strength. This step is aimed at the removal of contaminants such as producer-cell proteins and serum-derived proteins such as bovine serum albumin. Gel filtration, also defined as size exclusion chromatography, relies on the inability of the large LV particles to be retained by matrix pores. This step is aimed at the removal of salts and small size contaminants such as DNA fragments, as well as at medium exchange. The resulting LV preparation, undergoes one sterilizing 0.2 µm filtration and aseptic filling. The purified vector preparation is stored at -80°C.

3.4.1 Transgene activity assay

Huh7 cells (HCC cell line) were transduced with 2 vector dilutions. Three days after transduction, 10⁵ transduced cells are seeded and conditioned medium is collected 48 hours later and concentrated by Amicon Ultra 50 kD gel filtration columns (Millipore). The retentate is tested for FIX activity by aPTT.

3.4.2 Total DNA contamination assay

The assay is based on the use of a commercial kit (Topac). This assay is an ultrasensitive fluorescent nucleic acid stain to detect and quantify small quantity of dsDNA. The assay detects small fragments of DNA. A standard curve of Lambda DNA is analyzed in each test and the range of quantification is between 25 ng/ml and 1.56 ng/ml. Standard curve, samples and controls are excited at 485 nm and fluorescence emission intensity is measured at 535 nm by spectrofluorometer. Fluorescence emission intensity is plotted versus DNA concentration.

3.4.3 Producer-cell protein contamination assay

The assay is based on the use of a commercial enzyme-linked immunosorbent assay (ELISA) kit for the measurement of 293 cell proteins (Cygnus Technologies). Briefly, samples, which may contain proteins derived from 293 or 293T cells, are reacted in microtiter strips coated with an affinity-purified capture antibody. A standard curve is seeded for each test run. A second horseradish peroxidase (HRP) enzyme-labelled anti-293 antibody is reacted simultaneously, resulting in the formation of a sandwich complex of solid-phase antibody 293 proteins enzyme labeled antibody. The microtiter strips are washed to remove any unbound reactants, then the substrate tetramethyl benzidine (TMB) is added to each well and the reaction is stopped by the addition of kit stop solution. The amount of hydrolysed substrate is read on a microtiter plate reader at 450nm and is directly proportional to the concentration of 293 proteins present.

3.4.4 Endotoxin contamination assay

The assay is based on a kinetic chromogenic method (Charles River) for detection and quantification of bacterial endotoxin. In the test, co-lyophilised limulus amebocyte lysate (LAL) and substrate reagent are mixed with test sample in a microplate and incubate in a reader at 37° C. Absorbance measurements are collected with time after addition of chromo LAL. The time (onset time) taken for a sample to reach a specified absorbance (onset OD) is calculated; and a standard curve, showing the linear correlation between the log onset time and the log concentration of standard endotoxin, is generated.

3.4.5 Sterility assay

This assay is carried out using an automated system (BacT/ALERT instrument). Test samples are inoculated in two bottles containing media for aerobic and anaerobic microorganisms. The sample is automatically monitored for the presence of CO₂ eventually produced by microorganisms by a photodetector, which records a shift of the media colour from blue/green to yellow.

3.4.6 RCL assay

The assay is performed according to a published method^{253, 254}. RCL assay has been validated for sensitivity and specificity. The permissive cells C8166-45 are cultivated in presence of the LV sample for 28 days to allow amplification of a potential RCL. The cultures are inoculated at high cell density to maximise the chances for an RCL to infect. To ensure sensitivity and cell viability for 7 days the culture is minimally diluted by addition of medium but no portion of the cells is discarded. After 7 days the cell suspension is splitted 1:4 every 3 or 4 days for a total of seven passages. The RCL detection is done by ELISA determination of the HIV p24 protein concentration in culture supernatant. Moreover, C8166-45 gDNA is extracted to verify the absence of VSV-G DNA by qPCR. For each lot, at least 5% of total vector production is tested, according to what is recommended by United States Food and Drug Administration (FDA) for testing of retroviral vector-based gene therapy products for replication-competent retrovirus (FDA-CBER Guidance for Industry, 2006). As test positive control, pseudotyped virus R8.7 is used: this is an engineered virus derived from HIV, attenuated by inactivation of the four accessory genes *vif*, *vpr*, *vpu* and *nef* and pseudotyped with VSV-G, as described²⁵⁴.

3.5 Cell cultures

Cell lines (293T, Huh7 and DH82) were maintained in Iscove's modified Dulbecco's medium (IMDM, Sigma) supplemented with 10% foetal bovine serum (FBS, Gibco), 1% glutamine, penicillin 100 IU/ml and streptomycin 100 µg/ml. Primary hepatocytes were purchased from Biopredic (France) according to a protocol approved by the San Raffaele Ethical Committee (TIGET-HPCT) and maintained in proprietary media

following manufacturer's instructions. All cells were maintained in a 5% CO₂ humidified atmosphere at 37° C. Cells were cultured using cell culture-treated plastic devices (Falcon). Sub-confluent adherent cell lines were washed with sterile PBS (Sigma) and detached with 0.05% trypsin, 4 mM EDTA in PBS.

3.6 Complement sensitivity assay

Serum samples were obtained from healthy human donors, a pool of canine serum or individual dogs (provided by Dr. T. Nichols, University of North Carolina) to be screened before LV administration. Sera were thawed and half of each serum sample was heated at 56° C for 1 h to inactivate the complement. PGK.GFP LV particles were diluted in IMDM 10% FBS. A measure of 30 µl of the vector was diluted 1:5 into normal or heat-inactivated serum (or IMDM 10% FBS as the no-serum control) and the mixture was incubated at 37° C for 1 h. Following the incubation, medium was added to the reaction and then serially diluted and used to transduce 293T cells for end-point titre determination (see 3.3.2). The titre value was divided by the titre determined for the vector mixed with medium (the no-serum control) and reported as the percentage of recovery of titre compared to this control.

3.7 *In vitro* experiments

DH82 cells were transduced at the indicated multiplicity of infection (MOI) and GFP expression was analysed by flow cytometry (BD Canto System) 1 week after transduction. Huh7 cells were transduced with ICLVs or IDLVs at MOI 0.5, 5 or 50 and 3 days and 2 weeks after transduction GFP expression was analyzed by flow cytometry (BD Canto System) and total DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen), according to manufacturer's instructions. Human and canine primary hepatocytes were transduced at the indicated MOI. After 1 week of culture, nuclei were stained with Hoechst 33258 (Sigma) and analyzed by live fluorescence microscopy; total DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen), according to manufacturer's instructions. VCN was determined using an *ad hoc* qPCR, which selectively amplifies the reverse transcribed vector genome (both integrated and non-integrated) discriminating it from plasmid carried over from the transient transfection (RT-LV; ΔU3 sense: 5'-TCACTCCCAACGAAGACAAGATC-3', gag antisense: 5'

GAGTCCTGCGTCGAGAGAG-3'). The amount of human DNA loaded in the reaction was quantified with a qPCR designed to amplify the human telomerase gene as described in 3.3.2.

3.8 MicroRNA analysis

Small RNAs were isolated from DH82 cells by mirVana™ miRNA Isolation Kit according to manufacturer's instructions (Ambion). For analysis of miRNA expression, the Applied Biosystems Taqman® microRNA Assay system was used, as described³⁵. For each miRNA analyzed, 5 ng of small RNA was subjected to RT-PCR according to manufacturer's instructions. To determine absolute copy number, a standard curve was generated using an SDS-PAGE purified RNA oligonucleotide corresponding to let-7a (Primm). Five ng of total yeast RNA were spiked with concentrations of oligonucleotide ranging from 10² to 10⁸ copies, and analyzed by the Taqman® microRNA Assay. Reactions were carried out in duplicate in an ABI Prism 7900.

3.9 Mice experiments

Founder C57BL/6 *F9* KO mice were obtained from the laboratory of Dr. Inder Verma at the Salk Institute²⁵⁵. Mice defective for type-I IFN receptor 129/SVEV (*Ifnar1*^{-/-}) mice were obtained from B&K Universal Limited. Wt C57BL/6 and Balb/c mice were purchased by Charles River. All the mice were maintained in specific-pathogen free conditions. Vector administration was carried out in adult (7-10 weeks old) mice by tail-vein injections. Mice were bled from the retro-orbital plexus using capillary tubes and blood was collected into 0.38% sodium citrate buffer, pH 7.4. Mice were anesthetised with tribromoethanol (Avertin) and euthanized by CO₂ inhalation at the expected time points. All animal procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee.

3.10 Dog experiments

Haemophilia B dogs (carrying a E379G single amino-acid substitution in the FIX protein) were maintained at the Francis Owen blood research laboratory, that has both Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and United States Department of Agriculture (USDA) approval as an animal research

facility. The facility provides for breeding, whelping, housing, treating, and performing the experiments in the dogs on site. Complete blood counts and platelet counts were performed on EDTA-anti coagulated blood with a cell counter (Heska) calibrated for canine cells. Serum liver enzymes were obtained from a commercial veterinarian source.

3.10.1 Portal vein administration

Prior to making an incision, cFIX levels are raised to at least 10 to 20% by infusing an appropriate amount of normal canine plasma. A skin incision is made from the xyphoid down to the lower abdomen (~10 cm) and then the abdominal cavity is opened via its muscle layers. A baseline liver biopsy is taken for control tissue to determine VCN or gene expression. A small wedge of liver tissue is encircled with suture material and the tissue is processed for molecular and histological studies. The spleen is carefully exteriorized and a 4 or 5 French balloon tipped catheter is prepared by inflating the balloon for leaks (~0.5 to 1.0 ml). An appropriately sized branch of the splenic vein is isolated, circumferential sutures are positioned proximally and distally over about 2 cm, this branch of the splenic vein is punctured, the balloon tipped catheter is inserted and advanced into the portal vein, usually between 10 and 20 cm. The position of the catheter in the portal vein is confirmed visually or by palpation. The distal port of the balloon tipped catheter is aspirated to confirm blood can be withdrawn even when the balloon is securely inflate and occluding the portal vein. When the catheter position is optimised, a small amount (~1 ml) of vector is infused while the balloon is inflated. Infusion rates are adjusted as the blood pressure tolerates. After the vector is infused, the catheter is removed and the branch of the splenic vein is ligated proximally and distally and the spleen is returned to the abdomen. The surgical incision is closed in layers using suture material.

3.11 Protein immunisation and antigen re-challenge

Protein immunisation in naïve haemophilia B mice was carried out by subcutaneous administration of 50 µg of recombinant hFIX protein (Pfizer) in incomplete Freund adjuvant (IFA, Sigma). Antigen re-challenge was carried by subcutaneous administration of 5 µg of cFIX protein (Innovative Research) in IFA or by i.m. DNA

vaccination of a GFP-expressing plasmid, as described⁷⁶. Briefly, 0.5 nmol cardiotoxin-1 (Sigma) is injected in triadic leg muscles. Five days later mice are injected again in the same position with 50 µg/leg of pCCLsin.cPPT.CMV.GFP.wpre plasmid.

3.12 FIX, anti-FIX antibodies and canine cytokines ELISA

The concentration of hFIX or cFIX was determined in mouse or dog plasma by ELISA specific for hFIX or cFIX antigen respectively (Asserachrome, Affinity Biologicals, respectively) according to manufacturer's instructions. Absorbance of each sample is determined spectrophotometrically, using ELx800 automated microplate reader (BIO TEK Instruments) and normalised to antigen standard curves. The lower limit of quantification of the cFIX ELISA is 1 ng/ml or 0.02% of normal FIX levels, defined as the mean of 4 independent blank samples plus 3 standard deviations.

Mouse plasma samples were tested for the presence of anti-hFIX or anti-cFIX antibodies by ELISA as described⁴¹. Briefly, microtitre plates are coated with recombinant hFIX (Pfizer) or cFIX (Innovative Reseach) at 0.2 µg/well in 0.1 M carbonate buffer, pH 9.6. Two dilutions (1:100 and 1:1000) of mouse plasma are added and antibodies are detected with HRP-conjugated rabbit anti-mouse Ig (Dako). Plates are reacted with H₂O₂ and ortho-phenylenediamine and read at 492 nm. Plasma collected from haemophilia B mice previously immunised by subcutaneous administration of 50 µg of hFIX or cFIX antigen in IFA was used as a positive control for antibodies.

Canine cytokines concentration were determined in serum from treated and untreated dogs by a multiplex ELISA (Milliplex canine cytokine assay, Millipore), according to manufacturer's instructions.

3.13 Coagulation assays

Whole blood clotting times (WBCT) were performed on blood collected from treated dogs by a two-tube procedure at 28° C²⁵⁶. One ml of whole blood collected with a 1-ml syringe is distributed equally between two siliconised tubes (Vacutainer, Becton-Dickinson). The first tube is tilted every 30 sec. After a clot forms, the second tube is tilted and observed every 30 sec. The endpoint is the clotting time of the second tube. Canine FIX activity was determined in dog samples by aPTT, as described²⁵⁶. The

variability of the assay has been reported²⁵⁶.

Canine FIX activity was determined in mouse samples by a modified aPTT assay using canine FIX-deficient plasma (kindly provided by Dr. T. Nichols, University of North Carolina) and a semiautomated coagulometer (BioMerieux). Calibration curves were constructed with serial dilutions of pooled normal canine plasma (kindly provided by Dr. T. Nichols, University of North Carolina) and arbitrarily assigned a value of 100% FIX antigen or activity. Alternatively, cFIX activity was determined using a chromogenic assay, according to manufacturer's instructions (Hyphen Biomed), using serially diluted cFIX standards for calibration.

FIX inhibitor was determined by the Nijmegen modification of Bethesda assay²⁵⁷. The basic procedure is a 2-hour incubation of the test sample with Imidazole buffered (pH 7.4) normal canine pooled plasma with FIX activity of 100%. A reference mixture of test-specific normal canine pooled plasma with cFIX-deficient plasma is also prepared. After incubation the residual FIX activity (the relative percentage of FIX activity in the test sample mixture compared to the reference mixture) is converted into inhibitor Units/ml, where one inhibitor Unit is defined as the amount of inhibitor that yields 50% residual FIX activity. Inhibitor activity of test sample is read from a semi-logarithmic plot representing the correlation between residual FIX activity (logarithmic) and inhibitor activity (linear). The regression line is fully defined by 100% residual FIX activity with no inhibitor and 50% residual FIX activity with 1U inhibitor/ml.

3.14 Tail-clipping assay

Tail-clipping assay was performed as described²²³. Mice were anaesthetised and tail was placed in pre-warmed 37° C normal saline solution for 2 minutes and subsequently cut at 2.5-3 mm diameter. Tail was then immediately placed in 37° C normal saline solution and monitored for bleeding or clotting for 10 minutes. Blood-containing saline was centrifuged at 520 g for 10 min at 4° C to collect erythrocytes and resuspended in 6 ml of lysis buffer (10 mM KHCO₃, 150 mM NH₄Cl, 0.1 mM EDTA). Lysis proceeded for 10 minutes at room temperature and samples were centrifuged as above. OD at 575 nm of supernatants was measured.

3.15 Fractionation and sorting of liver cell populations

Mice were anesthetised with tribromoethanol (Avertin) and the abdomen and thorax were exposed. The liver was perfused (2.5 ml/min) *via* the inferior vena cava with 12.5 ml of the following solutions: 1) PBS EDTA (0.5 mM), 2) HBSS (Hank's balanced salt solution, Gibco-Life Technologies) and HEPES (10 mM), 3) HBSS-HEPES 0.03% Collagenase IV (Sigma). The dissociated liver tissue was harvested and smashed, passed through a 100 µm cell strainer (BD-Falcon) and processed into a single-cell suspension. This suspension was subsequently centrifuged three times at 35, 30 and 25 g to obtain hepatocyte-containing pellets. The non-parenchymal cells (nPC)-containing supernatant was loaded into a 30-60% Percoll (Sigma) gradient, centrifuged at 500 g and the nPC interface was collected. The nPC suspension was subsequently incubated with the following monoclonal antibodies: Fluorescein Isothiocyanate (FITC)-conjugated anti-CD45, Allophycocyanin (APC)-conjugated anti-F4/80, phycoerythrin (PE)-conjugated anti-CD31, APC-Cy7-conjugated anti-B220 (all from BD Biosciences). nPC subpopulations were sorted by fluorescence-activated cell sorting (FACS; MOFLO-DAKO-Beckman Coulter) according to the following markers: LSEC: CD45⁻ CD31⁺; KC CD45⁺ F4/80⁺ B220^{low}.

3.16 T- and B-cell assays and adoptive transfer

Mice were euthanized at the expected time point, spleens, livers and lymph nodes were collected and processed into single-cell suspension. Splenic CD8⁺ T cells were magnetically isolated from splenocytes by negative selection kit (Miltenyi Biotec). Intra-hepatic leukocytes (IHLs), which include T cell infiltrates, were isolated from the liver by smashing the tissue and running the sample on a Percoll (Sigma) gradient, as previously described⁷⁶. IFN-γ-secreting cells were enumerated by enzyme-linked immunospot (ELISPOT) assay in response to GFP-expressing cells as described⁷⁶. Splenocytes and IHLs were incubated with the following monoclonal antibodies: Allophycocyanin (APC)-conjugated Foxp3 staining kit (e-Bioscience); R-phycoerythrin (PE)-conjugated anti-CD25, Peridinin Chlorophyll (PerCP)-conjugated anti-CD8a; Pacific Blue-conjugated anti-CD4 (all from BD Biosciences). Detection of GFP-specific CD8⁺ T cells was performed by APC-labelled Pro5 MHC pentamer H-2K^d HYLSTQSAL (GFP₂₀₀₋₂₀₈) according to manufacturer's instructions (Proimmune).

Anti-FIX antibody secreting B cells were enumerated by ELISPOT culturing lymph

node, spleen and bone marrow cells for 24 hours. Lymph node cells were incubated with the following monoclonal antibodies: FITC-conjugated anti-CD19, PerCp-conjugated anti-B220, PB-conjugated anti-IgD, PE-Cy7-conjugated anti-IgM (all from BD Biosciences), Alexa647-conjugated recombinant hFIX (Pfizer). The conjugation reaction was performed by Alexa647 carboxylic acid, succinimidyl ester (Molecular Probe), according to manufacturer's instructions. Stained cells were analyzed with a FACSCanto flow cytometer equipped with Diva software (BD Biosciences). FIX-specific memory B cells were identified as CD19⁺ B220⁺ IgD⁻ IgM⁻ FIX-Alexa647⁺ cells.

C57Bl/6 OT-II TCR transgenic Ly5.2 mice were crossed with C57Bl/6 FOXP3-GFP knock-in Ly5.2 mice. FACS-sorted Ly5.2 OT-II CD4⁺ FOXP3-GFP⁻ were administered into C57Bl/6 Ly5.1 recipient mice by tail vein injection (2.5×10^6 cells/mouse), one day before OVA-encoding IDLV or IDLV.142T administration. Reconstitution of Rag2^{-/-}γ-chain^{-/-} mice was performed by intraperitoneal injection of pooled splenocytes and liver lymphocytes (6×10^7 cells/mouse) isolated from naïve, IDLV-treated or IDLV-142T-treated as described above.

3.17 Tissue analysis

The liver was fixed in 4% paraformaldehyde, embedded in optimal cutting temperature (OCT) and frozen in iso-pentane pre-cooled in liquid nitrogen. Cryostat sections (10 μm thick) were blocked with 5% FBS (Gibco), 1% bovine serum albumine, 0.1% Triton X-100 in PBS, and either directly analyzed under a 3-laser confocal microscope (Radiance 2100, Bio-Rad), or previously incubated with rabbit anti-GFP (Molecular Probe) washed and incubated with (FITC)-conjugated goat anti-rabbit Ig. Sections from untreated mice were used as negative controls. Nuclei were stained with TOPRO-3 (Molecular Probe).

3.18 Histopathology analysis

Histopathological evaluation was performed on liver, spleen, kidneys, heart, lungs and brain of mice treated with LVs expressing R338L or wt cFIX and on liver from one treated dog. Mice were euthanized at 1 year of age and approximately 10 months after LV administration, and organs were harvested, fixed in formalin and embedded in

paraffin. Canine liver was obtained through a surgical biopsy carried out 16 months after LV administration. One randomly collected 3 mm section for each mouse organ and 10 sections from canine liver were stained with haematoxylin and eosin and scored for any pathological sign.

3.19 VCN determination

Vector DNA was quantified as follows: gDNA was extracted from liver samples or fractionated/sorted liver cells by using Maxwell 16 Tissue DNA Purification Kit (Promega), or DNeasy Blood & Tissue Kit (Qiagen), respectively, according to manufacturer's instructions. VCN was quantified by qPCR using primers (HIV sense: 5'-TACTGACGCTCTCGCACC-3'; HIV antisense: 5'-TCTCGACGCAGGACTCG-3') and a probe (FAM 5'-ATCTCTCTCCTTCTAGCCTC-3') against the primer binding site region of LVs.. Endogenous DNA amount was quantified by a primer/probe set against the murine β -actin gene (β -Act sense: 5'-AGAGGGAAATCGTGCGTGAC-3'; β -Act antisense: 5'-CAATAGTGATGACCTGGCCGT-3'; β -Act probe: VIC 5'-CACTGCCGCATCCTCTTCCTCCC-3'). Copies per genome were calculated as described (3.3.2) The standard curve was generated by using samples with previously determined copies by Southern blot analysis.

3.20 Integration site analysis

To analyze ICLV and IDLV integrations we used standard and non-restrictive 5'- and 3'-LTR mediated linear-amplification mediated (LAM)-PCR^{258, 259}. Briefly, 100ng (for standard) or 500ng (for non-restrictive) DNA samples were pre-amplified by linear PCR using biotinylated primers hybridizing to LTR sequences: 5'-TTAGCCAGAGAGCTCCCAGG-3' for 5'-LTR LAM-PCR and 5'-AGCTTGCCTTGAGTGCTTCA-3' for 3'-LTR LAM-PCR. In combination with the previously reported linker cassette primers, two additional exponential amplifications were achieved using the following LTR primers, respectively: 5'-GATCTGGTCTAACCAGAGAG-3' and 5'-CCCAGTACAAGCAAAAAGCAG-3' for 5'-LTR LAM-PCR; 5'-AGTAGTGTGTGCCCCGTCTGT-3' and 5'-GATCCCTCAGACCCTTTTAGTC-3' for 3'-LTR LAM-PCR. LAM-PCR amplicons

were purified, tagged for downstream 454 pyrosequencing and bioinformatical analyses as previously described²⁴. Because of possible deletions in the LTR of the IDLV an amplicon sequence was considered as valid vector-host genome junction if the megaprimer sequence and at least 5 or 18 nucleotides of the LTR were present. The remaining sequences were automatically aligned to the mouse genome (assembly NCBI37/mm9) using BLAT²⁶⁰. Alignments with at least 95% sequence identity were reported as integration sites.

3.21 Statistical analysis

Standard statistical analyses were performed using Student t-test and ANOVA in experiment comparing variables at each time-point at $\alpha=0.05$ level of confidence.

In order to test differences in transgene expression or clotting activity over time, instead we applied a statistical methodology within a nonparametric permutation setting.

4.RESULTS

4.1 Evaluating LV-mediated liver gene therapy in the haemophilia B canine model

Haemophilic dogs are valuable models for stringently evaluating the efficacy, acute and long-term toxicity and immunogenicity of new therapeutic strategies. They also provide a more realistic and predictive model to assess dose-response as compared to mice. The haemophilia B dogs of the Chapel Hill colony (North Carolina, USA) have a spontaneous missense mutation that causes a single amino-acid substitution in the FIX protein (E379G), leading to impaired protein folding and secretion into the bloodstream²⁶¹. These dogs are thus completely devoid of FIX activity in the circulation and closely recapitulate the human disease phenotype. They undergo spontaneous bleedings 5.5 times a year on average and are cared by on-demand plasma transfusions from normal dogs. We set out to translate and evaluate in this model our LV-mediated liver gene therapy approach that proved effective in haemophilia B mice⁷³.

4.1.1 LVs efficiently transduce canine hepatocytes, the activity of ET promoter and miR-142 is preserved in canine cells and canine serum minimally inactivate LV particles

We first aimed to address issues that may negatively affect the feasibility of translation of our LV-mediated liver gene therapy strategy to dogs. We first generated a LV expressing the specie-specific cFIX transgene under the control of the synthetic hepatocyte-specific promoter designated as ET (Enhanced Transthyretin)²⁶² and carrying target sequences for miR-142 (ET.cFIX.142T, Figure 1). In order to verify *in vivo* transgene activity, we intravenously injected 5×10^8 transducing units (TU, determined by end-point titration on a permissive cell line), corresponding to approximately 10 µg of HIV Gag p24 equivalents (p24) of ET.cFIX.142T in haemophilia B mice (n=2). In both mice we measured approximately 5% of normal cFIX activity over time in the plasma of treated mice, thus confirming transgene functionality (Figure 2A).

Figure 1

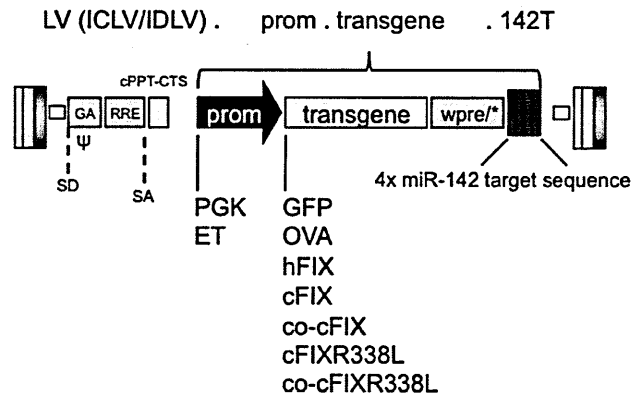


Figure 1: Schematic of LVs used in this work. Schematic representation of the third-generation self-inactivating LVs (provirus) used in this work and how they are referred to as in text. SD: splicing donor site. SA: splicing acceptor site. ψ : packaging signal, including 5' portion of GAG gene (GA). RRE: Rev responsive element. cPPT-CTS: central polypurine tract to central termination sequence. wpre/wpre*: woodchuck hepatitis virus post-regulatory element/mutated wpre (see 1.4). 142T: miR-142 target sequence made of 4 tandem copies of a sequence perfectly complementary to miR-142. Promoters used: ubiquitously expressed phosphoglycerokinase (PGK) promoter, hepatocyte-specific enhanced transthyretin (ET) promoter composed of synthetic hepatocyte-specific enhancers and transthyretin promoter. Transgenes used: green fluorescent protein (GFP), chicken ovalbumin (OVA), wt human or canine coagulation Factor IX (hFIX, cFIX), codon-optimised cFIX (co-cFIX), hyper-active cFIX (cFIXR338L), codon-optimised hyper-active cFIX (co-cFIXR338L). Vectors were produced with integrase-competent (ICLV) or integrase-defective (IDLV) packaging constructs (see 1.4.2). Unless otherwise indicated LVs were produced with canonical integrase-competent packaging constructs. Unless otherwise indicated LVs were pseudotyped with the VSV-G envelope.

It has been shown that mouse serum has little effect on the stability of LV particles in the circulation, whereas human and NHP serum may inactivate vector particles through the complement system^{263, 264}. To determine the extent of complement-mediated inactivation, LV titres were measured after exposure to canine serum and the results reported as percentage of recovery of titre compared to control samples not exposed to serum (Figure 2B). LV displayed some sensitivity to canine serum with 2- to 5-fold reduction in titre and some inter-individual variability. Pre-incubation of LV with the serum obtained from 2 healthy human donors resulted in 10-fold titre reduction. These data show that there is no major inactivation of LV in canine serum. However, we

decided to pre-screen serum from 3 candidate dogs and select for infusion the dog whose serum displayed the lowest neutralizing potential against LV.

Figure 2

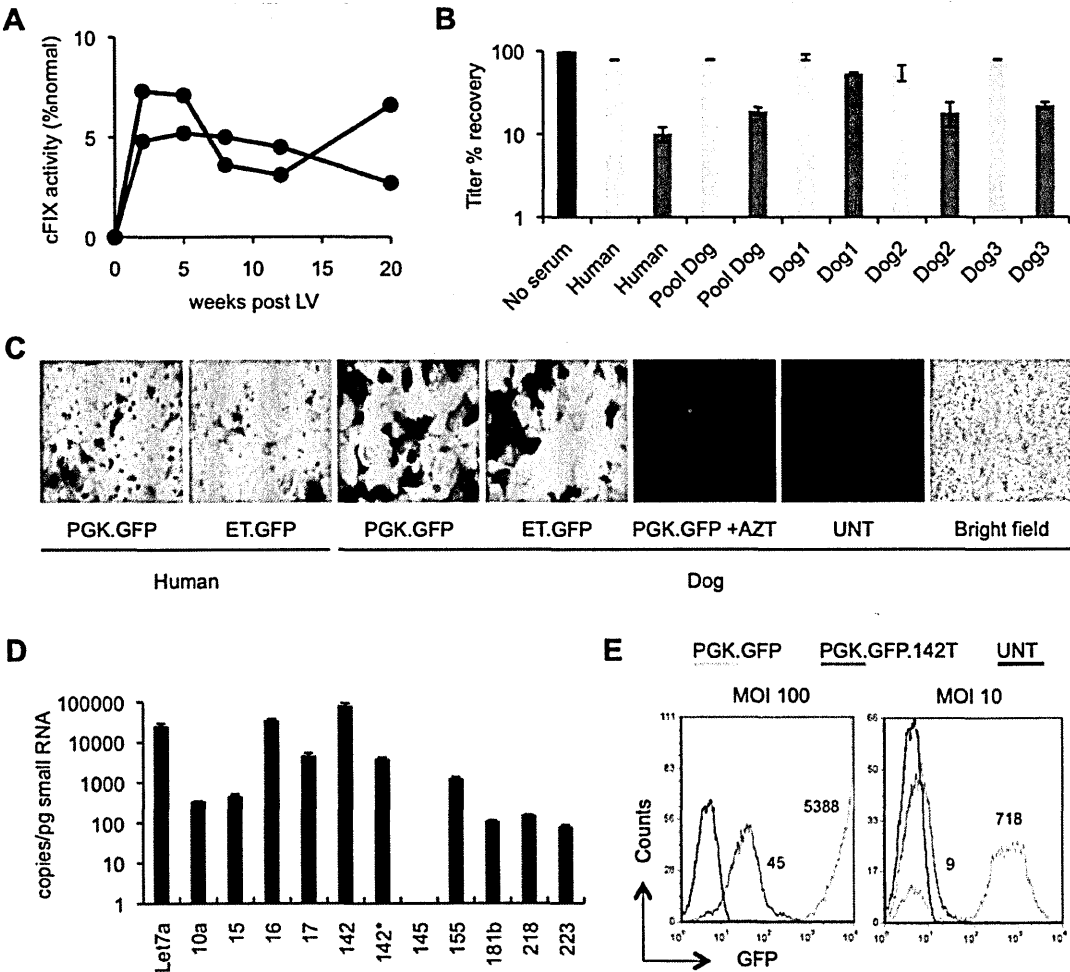


Figure 2: Feasibility of translation of LV-mediated liver gene therapy to dogs. (A) cFIX activity measured by aPTT in plasma collected at the indicated times after LV administration in haemophilia B mice. Results are presented for individual mice. (B) Percentage of titre remaining after LV incubation with normal (dark grey bars) or heat-inactivated (light grey bars) serum, compared to no serum control, set as 100% recovery. PGK.GFP LVs were incubated for 1 h at 37° C with medium (no serum control) or serum of 2 healthy human donors, 3 different dogs or a pool of canine serum. Results are presented as mean±range of 2 independent experiments. (C) Representative images of primary human or canine hepatocytes cultures transduced with the indicated LVs (MOI 100) or left untransduced (UNT) and analysed 3 days after transduction by live fluorescence microscopy. The reverse transcriptase inhibitor

azidothymidine (AZT) was used as a control for pseudotransduction. (D) miRNA levels in DH82 cells were quantitated by RT-qPCR using primers and probe specific for the indicated miRNAs. Results are presented as mean±standard error of the mean (SEM) of different RNA extractions (n=5). (E) Histogram and GFP mean fluorescence intensity (MFI) of flow cytometry analysis of DH82 cells transduced with PGK.GFP (light grey line) or PGK.GFP.142T (dark grey line) LV at the indicated MOI, or left UNT (black line).

To verify promoter activity in canine hepatocytes and exclude canine-specific restriction to LV transduction, we transduced primary human and canine hepatocytes *in vitro* at multiplicity of infection (MOI) 100 with LVs expressing GFP under the control of the ubiquitously expressed phosphoglycerokinase (PGK) promoter or the ET promoter. We observed high levels of transduction and transgene expression in hepatocytes derived from both species (Figure 2C). Interestingly primary human hepatocytes were transduced better than canine hepatocytes.

The miR-142 sequence is conserved between human and dog. To ensure that miR-142-mediated regulation would be reproduced in dogs, we assessed the conservation of miR-142 expression and activity in canine hematopoietic-lineage cells. Expression levels of selected microRNAs were analyzed in DH82 cells, a cell line derived from canine macrophages, using a quantitative reverse-transcription PCR (Figure 2D). The expression patterns resembles that of U937 cells, a human monocytic cell line, demonstrating cross-species conservation of many microRNAs³⁵. Importantly, miR-142 was found to be expressed at high levels. To examine miR-142 activity on transgene suppression, DH82 cells were transduced with reporter LVs encoding GFP with or without regulation by miR-142, and GFP expression was analyzed by flow cytometry. As shown in figure 2E, canine miR-142 mediated down-regulation of GFP expression more than 100-fold and to levels comparable with the negative population when low vector concentration was used.

Taken together, these data indicate that cFIX transgene cloned in the vector is functional, the ET promoter and miR-142T activity are preserved in cells of canine origin and that the extent of LVs inactivation in canine serum is not incompatible with their *in vivo* administration. Based on these promising data we proceeded with large-scale manufacturing and testing of ET.cFIX.142T LV in the haemophilia B canine model.

4.1.2 LV-mediated liver gene therapy is safe and feasible in a haemophilia B dog and allows long-term transgene expression

Taking advantage of a manufacturing process previously validated for the production of clinical-grade LVs, we produced a large-scale batch (2009/D2) of LV qualified for *in vivo* administration. The process consists in quadritransfection of a producer 293T cells bank, collection of vector-containing supernatants, benzonase treatment to reduce plasmid DNA carryover, followed by 2 steps of column chromatography (an ion exchange and gel filtration) and sterilising filtration, with a overall yield of purified LV particles of approximately 25% of the initial output (Figure 3). A similar process has been recently reported²⁵². More details can be found in 3.4. Quality assessment of vector preparation is summarised in Table 1. The process yielded a total of 1.1×10^{10} TU, corresponding to 864 μg p24 in 160 ml PBS for infusion. The LV had an infectivity of 1.3×10^4 TU/ng p24. The preparation was tested for the absence of RCL. The preparation had a low endotoxin content, compatible with i.v. administration in dogs (maximal tolerated dose 5 endotoxin unit/kg/hour) and was sterile. We treated an 8-month old, 20-kg male haemophilia B dog (M57) by intraportal administration of the whole LV lot described above (Table 1), corresponding to a dose of 5.7×10^8 TU/kg and 44 μg p24/kg.

The infusion was well tolerated and uneventful, except for a transient rise in body temperature, which returned to normal after 1 day. We determined aminotransferases (alanine aminotransferase, ALT and aspartate aminotransferase, AST) levels in serum collected from the treated dog in the first days after LV administration (Figure 4A,B). Both ALT and AST increased 6 and 11 fold respectively as compared to baseline, reaching values slightly above the normal range for 3 and 1 day, respectively (250 and 200 U/l), indicating a minor and self-limiting hepatocellular toxicity. Platelet counts were slightly below the normal range for 6 days after LV administration (Figure 4C). These events were asymptomatic and did not require intervention. Since haematocrit was also low (32%, normal range 37-55%), the minor reduction in platelet counts is consistent with haemodilution, as the dog received >500 ml of liquids during the infusion procedure (200 ml of normal dog plasma transfusions both before and after surgery and the LV volume). All the other blood chemistry parameters were in the normal range. We also monitored serum levels of some canine cytokines (IL-2, IL-4,

IL-6, IL-7, IL-8, IL-10, IL-15, TNF- α , TGF- β , IFN- γ) for the first 50 days after LV administration and found a sharp induction of TNF- α , IL-6 and IL-8 levels since the first hours after LV administration, rapidly declining over the following hours, consistent with an acute inflammatory response to LV particles (Figure 4D-F). All the other cytokines were found at levels not significantly different from control untreated animals.

Figure 3

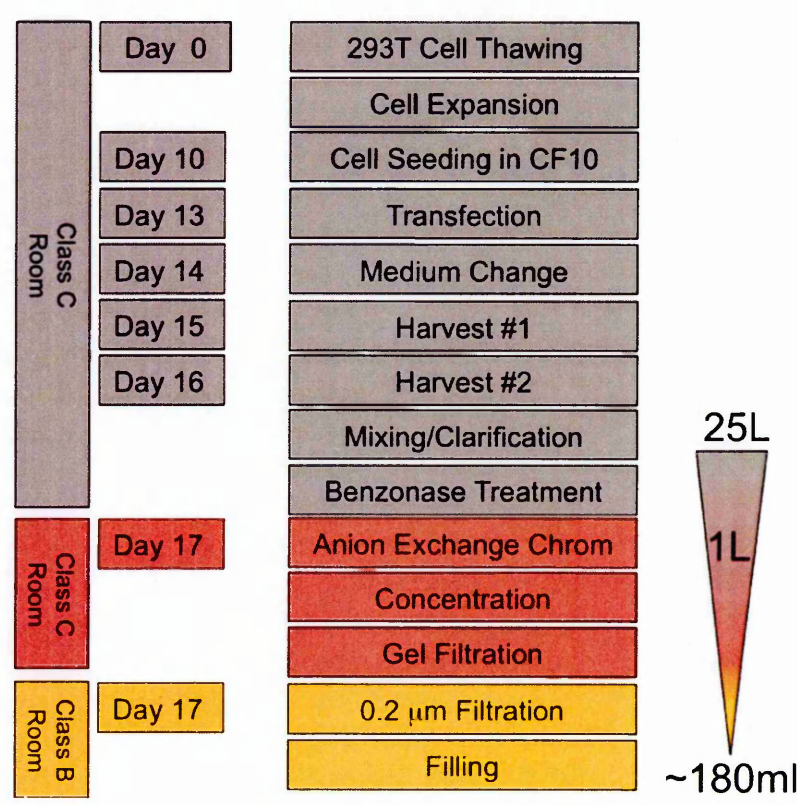


Figure 3: Flow chart of large-scale LV production. CF10: 10-tray cell factories. Further details can be found in 3.4

Table 1. Quality controls of the large-scale purified LV Batch 09/D2

Batch 2009/D2			Specification
Particles	μ g p24/ml	5.4	For information only
Titre	TU/ml	7.0×10^7	$>1.0 \times 10^8$
Infectivity	TU/ng p24	1.3×10^4	$>1.0 \times 10^4$
Transgene Activity		Positive	Positive

Total DNA	µg/ml	2.6	For information only
Producer cell protein	ng/ml	82	For information only
Endotoxin	EU/ml	0.3	<1
Sterility		Negative	Negative
RCL		Negative	Negative
Volume	ml	160	
Particles	Total µg p24	864	
Titre	Total TU	1.1x10 ¹⁰	
Endotoxin	Total EU	48	

The table shows the results of selected quality control assays performed on the large-scale purified LV Batch 2009/D2. The specifications for GMP-grade LV production are listed. Please note that this batch is a research-grade production. EU: endotoxin units. TU: transducing units.

Figure 4

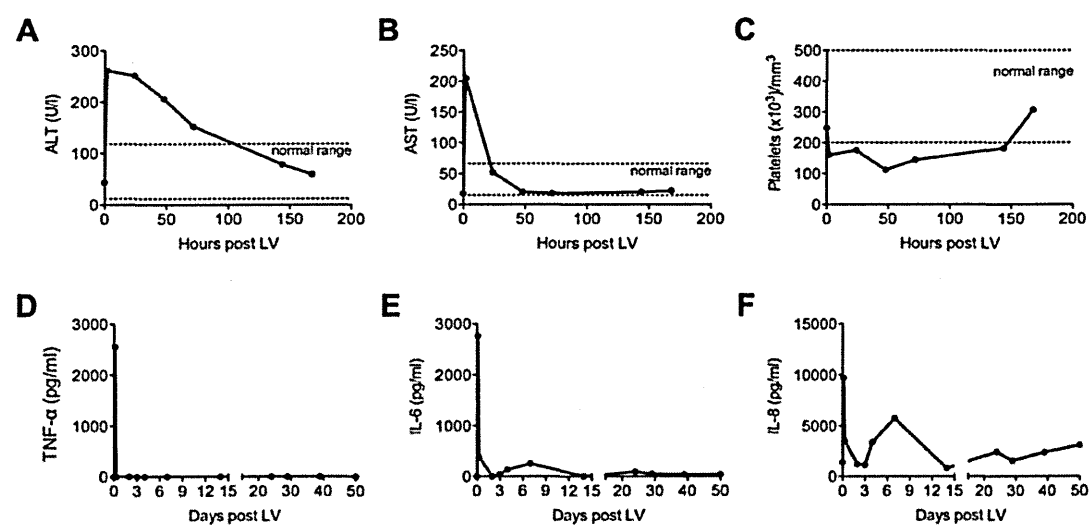


Figure 4: Acute toxicity and cytokines levels after portal vein infusion of LV.ET.cFIX.142T in the haemophilia B dog (wt transgene). ALT (A) and AST (B) serum levels, blood platelets counts (C), TNF-α, (D), IL-6 (E) and IL-8 (F) serum levels measured in samples collected at the indicated times after LV administration.

We measured the whole blood clotting time (WBCT), aPTT and cFIX antigen levels in blood or plasma samples collected from dog M57 at routine intervals after LV administration. At the current follow-up (>3 years after treatment) the dog is alive and well. Both WBCT and aPTT were stably shortened, albeit without reaching normal levels (Figure 5A,B) and anti-cFIX inhibitors tested negative in two independent

determinations (at day 7 and 50 after LV administration). We found detectable cFIX antigen levels, ranging from 0.5 to 4.5 ng/ml, corresponding to 0.01 to 0.09% of normal levels (Figure 5C). We calculated cFIX activity based on a curve correlating cFIX levels and WBCT or aPTT²⁵⁶ and found that cFIX activity averaged 0.1% of normal and ranged from 0.01 to 0.7% of normal (Table 2). Although the reconstituted activity was low, the frequency of spontaneous bleeding events was significantly lower as compared to the expected, as the dog experienced only 3 out of 17 spontaneous bleedings expected for the time of observation after gene therapy. A liver biopsy was taken at 16 months after LV administration and resulted negative for any pathological abnormality.

Figure 5

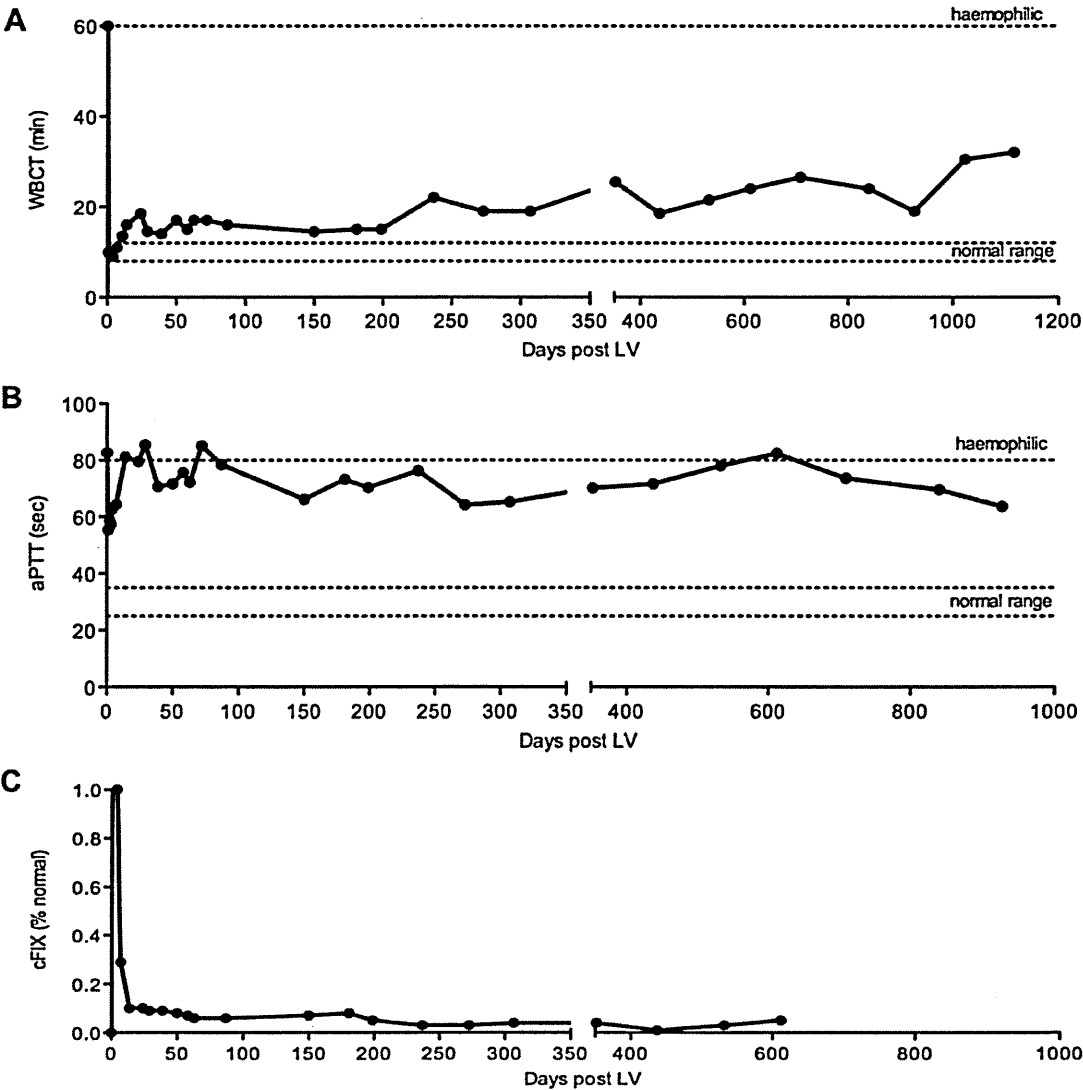


Figure 5: Efficacy of liver gene therapy with LV.ET.cFIX.142T in the treated haemophilia B dog (wt transgene). WBCT (A), aPTT (B) and cFIX antigen levels measured on blood or plasma samples collected at the indicated times after LV administration.

Table 2. Dose of LV and treatment efficacy in the haemophilia B dog treated with LV.ET.cFIX.142T (wt transgene)

DOG	M57 (Hemil)
TU/kg	5.7x10 ⁸
µg p24/kg	44
FU (days)	1116
WBCT (min)	20 (14-32)
WBCT-based cFIX activity (% normal)	0.15 (0.015-0.7)
aPTT (sec)	72.5 (63.6-85)
aPTT-based cFIX activity (% normal)	0.12 (0.01-0.2)
cFIX antigen (% normal)	0.05 (0.01-0.09)

The table shows the infused dose of LV (titre and particles) *per* weight, the follow up (FU) time, the results of the coagulation assays performed to determine cFIX activity and the cFIX antigen levels (determined by ELISA). Results are presented as mean of values over time (range of determined values).

Overall these data indicate that liver gene therapy by LVs in a dog affected by haemophilia B was feasible and provided long-term expression of cFIX with some therapeutic benefit, without eliciting anti-FIX immune responses. However, cFIX activity could only reach approximately 0.1% of normal levels at the dose infused. Thus, it is clear that an increase in LV dose and/or potency is needed to improve therapeutic efficacy.

4.2 Evaluating a codon-optimised FIX transgene in the haemophilia B mouse and dog model

Since increasing the LV dose to treat dogs represents a challenge for the current manufacturing capacity of large-scale purified vector batches, it becomes crucial to maximize vector potency. In order to increase vector potency we first evaluated whether increased transgene expression levels can be obtained *in vivo* by exploiting a codon-optimised cFIX transgene (co-cFIX). Codon-usage optimisation is aimed at maximizing the output of a transgene product by both stabilizing transgene mRNA and enhancing its translation. This process can be carried through bioinformatics tools and consists of substitutions of codons with synonymous ones that are the most frequently found in highly expressed genes in a species and are recognized by the most abundant tRNAs. Moreover, *cis*-acting elements that could impact on mRNA stability are eliminated or changed^{265, 266}.

4.2.1 A codon-optimised transgene increases FIX expression 2- to 3-fold in haemophilia B mice

We set out to evaluate the transgene expression levels achieved upon liver gene therapy with LV expressing the co-cFIX transgene in haemophilia B mice. This transgene was generated by changing 156 out of the total 452 triplets composing its cDNA (by a subcontractor). Within the modified codons, 140 were changed by third-base substitution, while 16 codons (including the STOP codon) were fully changed. We then generated a miR-142- regulated LV bearing this co-cFIX transgene under the transcriptional control of the ET promoter (ET.co-cFIX.142T, Figure 1).

We compared the potency of this improved expression cassette with that of the same cassette containing the wt cFIX transgene (ET.cFIX.142T), upon i.v. administration in haemophilia B mice. The titre and infectivity of these LVs were similar. We treated haemophilia B mice with two different matched doses (5 or 10x10⁸ TU/mouse) of ET.co-cFIX.142T or ET.cFIX.142T (n=4/group). We measured cFIX protein expression and clotting activity on plasma samples collected from treated mice at regular intervals after LV administration (Figure 6). We observed that cFIX activity was 3.4- and 2.2-fold higher for the co-cFIX than its wt counterpart in both dose cohorts, reaching up to 38.4 ± 4.8% (mean ± standard error of the mean, SEM) of normal clotting activity in the

high-dose group (Figure 6B,D). Vector copies *per* diploid genome (vector copy number, VCN) were similar in the liver of treated animals receiving matched doses of LVs bearing wt or co-cFIX, measured at the end of experiment. This result indicates that the observed increase in clotting activity was not due to increased transduction. FIX inhibitors were undetectable by Bethesda assay in all treated mice. These data show that a co-cFIX transgene can significantly increase expression, without any evident impairment of specific enzyme activity (as measurable by the ratio of activity to antigen) nor increased immunogenicity²⁶⁷. Based on these data, we next evaluated the use of this co-cFIX transgene in the haemophilia B canine model.

Figure 6

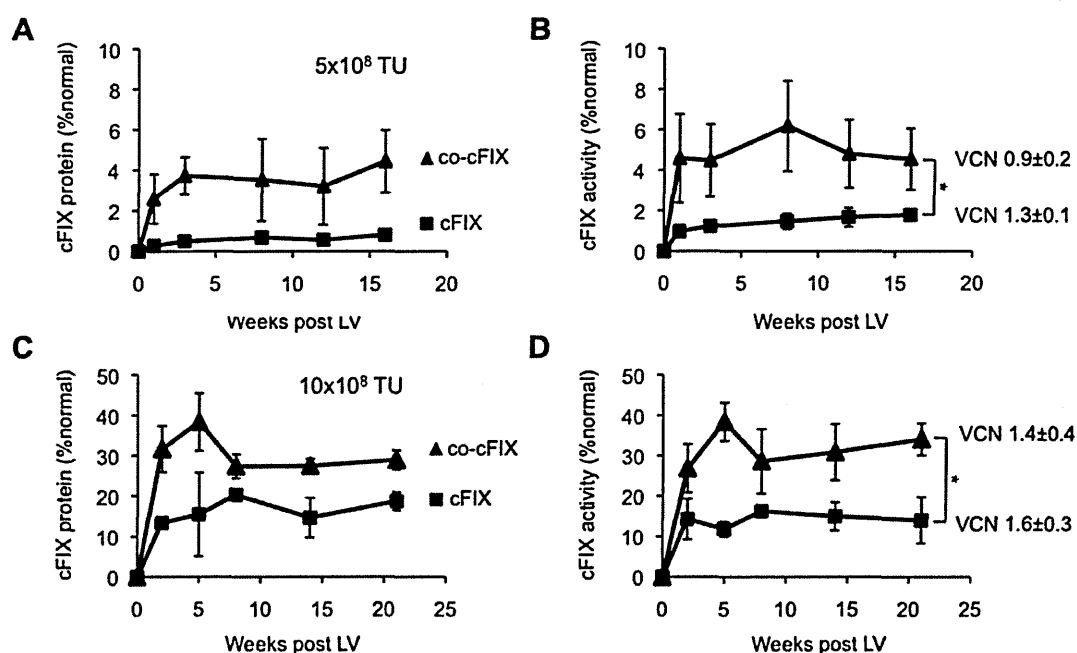


Figure 6: Codon-optimised cFIX transgene in haemophilia B mice. cFIX expression and clotting activity measured by ELISA (A,C) and aPTT (B,D) respectively on plasma samples collected from treated mice at the indicated times after LV administration. Mice were treated with 5×10^8 (A and B) or 10×10^8 (C and D) TU/mouse of ET.cFIX.142T (squares, $n=4$) or ET.co-cFIX.142T (triangles, $n=4$). VCN in genomic DNA from the liver of treated mice measured at the end of experiment is indicated. Results are presented as mean±SEM. *: p GLOBAL<0.05 (non parametric combination statistics).

4.2.2 LV-mediated liver gene therapy with a higher dose and a co-cFIX transgene reconstitutes higher FIX activity in a second haemophilia B dog

We produced a second large-scale batch (2011/D13-15) of purified LV carrying the co-cFIX transgene. In an effort to increase also the LV dose to administer to the dog, we used improved transfection and collection conditions to increase the LV yield. We also optimised the formulation medium by adding 5% DMSO to increase vector stability and reduce adsorptive loss during manipulation and administration. Quality assessment of the new vector preparation is summarised in Table 3. This LV batch had a 3.4-fold increased infectivity as compared to the previous batch (most probably due by the combination of process optimisation and the use of codon-optimised transgene), thus resulting in a 4-fold higher TU dose and only slightly higher p24 dose to be administered (4.5×10^{10} total TU, 1030 total μg p24, infectivity 4.4×10^4 TU/ng p24). The preparation had a low endotoxin content, compatible with i.v. administration in dogs and was sterile.

Table 3. Quality controls of the large-scale purified LV Batch 11/D13-15

Batch 2011/D13-15			Specification
Particles	μg p24/ml	6.3	For information only
Titre	TU/ml	2.7×10^8	$>1.0 \times 10^8$
Infectivity	TU/ng p24	4.4×10^4	$>1.0 \times 10^4$
Transgene Activity		Positive	Positive
Total DNA	$\mu\text{g}/\text{ml}$	1.4	For information only
Producer cell protein	ng/ml	221	For information only
Endotoxin	EU/ml	<0.8	<1
Sterility		Negative	Negative
RCL		Negative	Negative
Volume	ml	164	
Particles	Total μg p24	1030	
Titre	Total TU	4.5×10^{10}	
Endotoxin	Total EU	<137	

The table shows the results of selected quality control assays performed on the large-scale purified LV Batch 2011/D13-15. The specifications for GMP-grade LV production are listed. Please note that this batch is a research-grade production. EU: endotoxin units. TU: transducing units.

We treated a second haemophilia B dog (O21, male, 21-months old, 22 Kg of body weight) by intraportal administration of the whole LV preparation described in Table 3,

corresponding to a dose of 2.3×10^9 TU/Kg and 47 μg p24/Kg. During infusion, the dog experienced an unexpected acute hypotension attributed to a type-I hypersensitivity reaction to an unknown component of the vector batch. This event was successfully managed by immediate administration of an antihistamine drug (Benadryl 1 mg/kg, i.v.) and a corticosteroid (Dexamethasone 25 mg/kg i.v.). LV infusion was subsequently completed upon blood pressure recovery. We observed a transient rise in body temperature. ALT and AST serum levels increased 2 and 4 fold respectively as compared to baseline, resulting in levels slightly above the normal range for 3 and 1 day, respectively (160 and 120 U/l). This increase indicates a minor and self-limiting hepatocellular toxicity, similar or even lower to that observed in dog M57 (Figure 7A,B). Platelet counts were slightly below the normal range for 2 days after LV administration (Figure 7C). Since haematocrit was also low (26%), the minor reduction in platelet counts is consistent with haemodilution, as described above (4.1.2). All the other blood chemistry parameters were in the normal range.

Figure 7

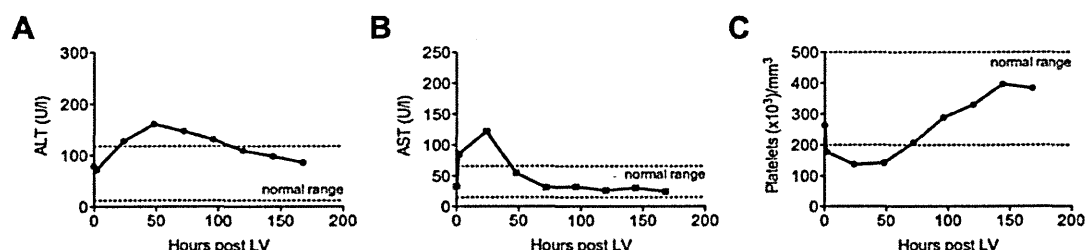


Figure 7: Acute toxicity after portal vein infusion of LV.ET.co-cFIX.142T in the haemophilia B dog (codon-optimised transgene). ALT (A) and AST (B) serum levels, blood platelets counts (C) measured in samples collected at the indicated times after LV administration.

The WBCT, aPTT and cFIX antigen levels were measured in blood or plasma samples collected from dog O21 at routine intervals after LV administration (Figure 8). At the current follow-up (>6 months after treatment) the dog is alive and well. We could measure cFIX antigen levels ranging from 20 to 40 ng/ml, corresponding to 0.4 to 0.8% of normal levels (Figure 8C). We determined cFIX activity based on the WBCT or aPTT²⁵⁶ and found that it ranged from 0.1 to 1.5% of normal (Table 4). O21 has not

experienced any spontaneous bleeding over the last 6 months, indicating a clinical benefit. Since this dog received a 4-fold higher LV dose (in TU) and showed approximately 10-fold higher cFIX antigen and activity with respect to the previously treated dog, transgene codon-usage optimisation may have increased FIX levels by 2.5-fold as compared with wt cFIX transgene, in line with the data obtained in the mouse model.

Figure 8

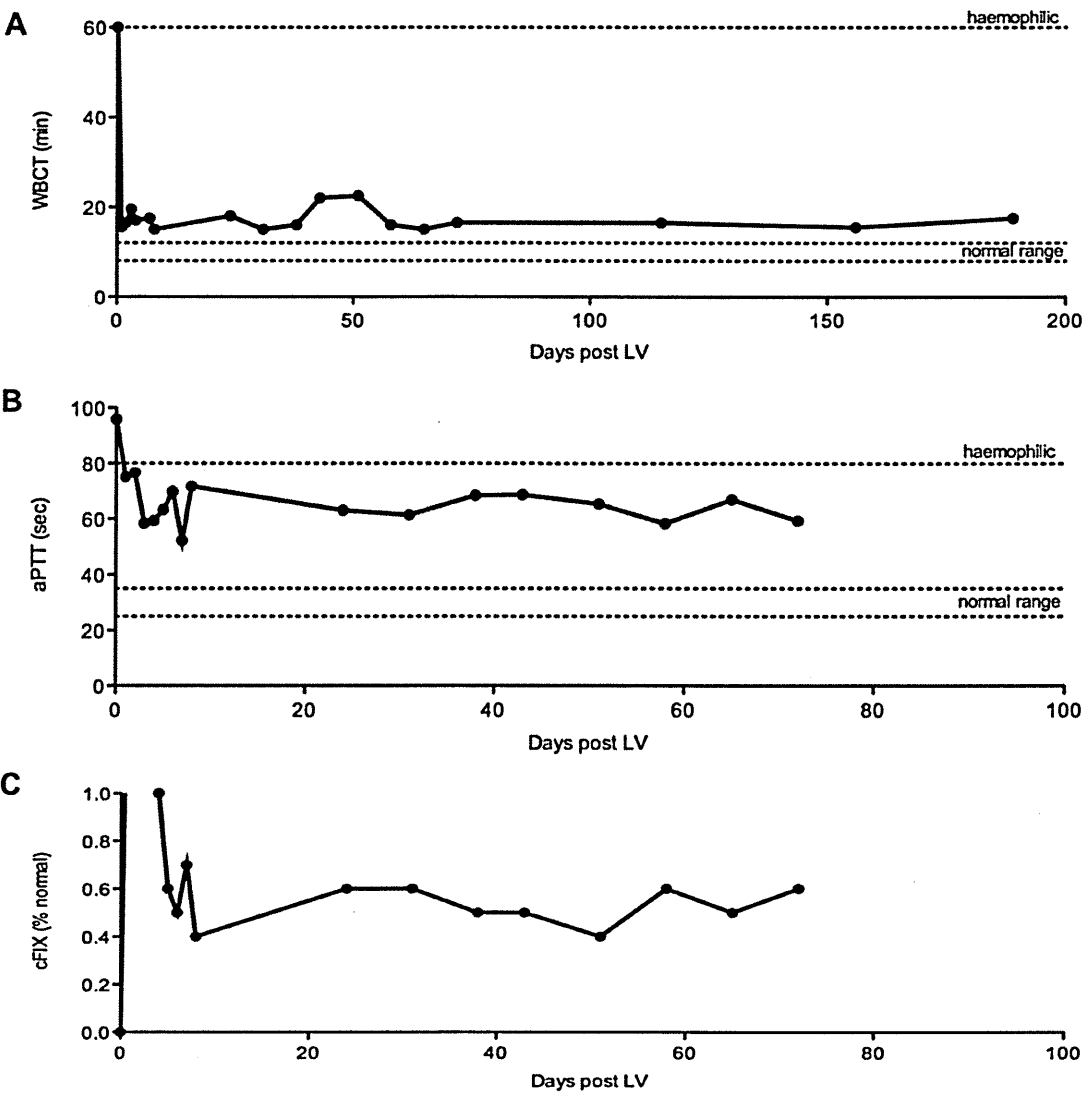


Figure 8: Efficacy of liver gene therapy with LV.ET.co-cFIX.142T in the treated haemophilia B dog (codon-optimised transgene). WBCT (A), aPTT (B) and cFIX

antigen levels measured on blood or plasma samples collected at the indicated times after LV administration.

Table 4. Dose of LV and treatment efficacy in the haemophilia B dog treated with LV.ET.co-cFIX.142T (codon-optimised transgene) in comparison with the previously treated dog (Table 2)

DOG	M57 (Hemil)	O21 (Valentine)
TU/kg	5.7x10 ⁸	2.3x10 ⁹
µg p24/kg	44	47
FU (days)	1116	189
WBCT (min)	20 (14-32)	17.3 (15-22.5)
WBCT-based cFIX activity (% normal)	0.15 (0.015-0.7)	0.4 (0.1-1.15)
aPTT (sec)	72.5 (63.6-85)	63.9 (58-68)
aPTT-based cFIX activity (% normal)	0.12 (0.01-0.2)	0.2 (0.18-1.5)
cFIX antigen (% normal)	0.05 (0.01-0.09)	0.6 (0.4-0.8)

The table shows the infused dose of LV (titre and particles) *per weight*, the follow up (FU) time, the results of the coagulation assays performed to determine cFIX activity and the cFIX antigen levels (determined by ELISA). Results are presented as mean of values over time (range of determined values).

4.3 A codon-optimised and hyper-active FIX transgene increases the potency of LVs after liver gene therapy in haemophilia B mice

In an effort to further increase vector potency, we evaluated the use of a hyper-functional FIX carrying a R338L amino acid substitution, previously associated with clotting hyperactivity and thrombophilia²⁶⁸. FIX-R338L is a naturally occurring mutant found in a young man with a diagnosis of deep-venous thrombosis and termed FIX Padua, after the city of patient origin. It was reported that the patient had >700% of normal FIX activity with normal FIX antigen levels. This mutation affects a residue that is crucial for FX binding and causes a gain-of-function phenotype conferring high risk

of thrombosis. Recombinant FIX-R338L was shown to have 5- to 10-fold higher specific activity with respect to wt FIX. While this mutant caused thrombophilia in a normal individual hemizygous for the mutation, its use in gene therapy for haemophilia B is attractive, as lower amounts of this hyper-functional FIX could potentially result in correction of the disease phenotype.

Therefore we generated wt or co-cFIX transgenes bearing a point mutation corresponding to the previously described FIX-R338L mutant. We cloned these transgenes in LVs bearing the hepatocyte-specific ET.142T expression cassette (Figure 1). As shown in Figure 9A, haemophilia B mice treated with LV carrying the wt (n=4) or hyper-functional FIX-R338L (n=6; 7×10^8 TU/mouse) expressed about 8% of normal cFIX antigen in the circulation, while mice treated with the hyper-functional co-cFIX-R338L transgene (n=7; 7×10^8 TU/mouse) reached about 20% (consistent with the aforementioned 2- to 3-fold increased expression of co-cFIX). However, both the hyper-functional cFIX-R338L and co-cFIX-R338L transgenes exhibited >5-fold higher activity with respect to the antigen levels, resulting in up to 125% of normal clotting activity for the co-cFIX-R338L transgene (Figure 9B). These data show that the hyper-functional co-cFIX-R338L transgene provides a >15-fold gain in potency with respect to the wt sequence and is greater than what has been reported with other FIX mutants^{206, 269}. These data were confirmed in the group of our collaborator Thierry VandenDriessche for analogous codon-optimised and R338L human FIX transgenes, following LV-mediated liver gene therapy in haemophilia B mice.

To evaluate whether the co-cFIX-R338L transgene allows lowering LV doses to reach therapeutic activity, haemophilia B mice were injected with 1.25 or 2.5×10^8 TU/mouse. Treated mice expressed about 0.7 and 3.4% of normal cFIX protein, respectively. However, their clotting activity was about 6.4 and 19% of normal, with 6- to 9-fold hyperactivity with respect to protein level (Figure 9C,D). None of the mice treated with hyper-functional transgenes developed inhibitors. In a tail-clipping assay, haemophilia B mice treated with low dose co-cFIX-R338L LV lost significantly less blood than mice treated with a matched dose of co-cFIX LV and were indistinguishable from wt mice, indicating the superior performance of the R338L FIX in achieving haemostasis *in vivo* (Figure 9E). To exclude that the use of this mutant impaired tolerance induction towards cFIX protein, haemophilia B mice were treated with 5×10^8

TU/mouse of co-cFIX or co-cFIX-R338L and subsequently subjected to immunisation with cFIX protein 6 weeks after LV administration. Mice treated with LVs expressing either transgene did not develop anti-hFIX antibodies, even after antigen re-challenge with cFIX protein, indicating that gene therapy induced immune tolerance to the wt protein (Figure 9F). In contrast, untreated haemophilia B mice developed anti-cFIX antibodies after immunisation with cFIX protein.

Figure 9

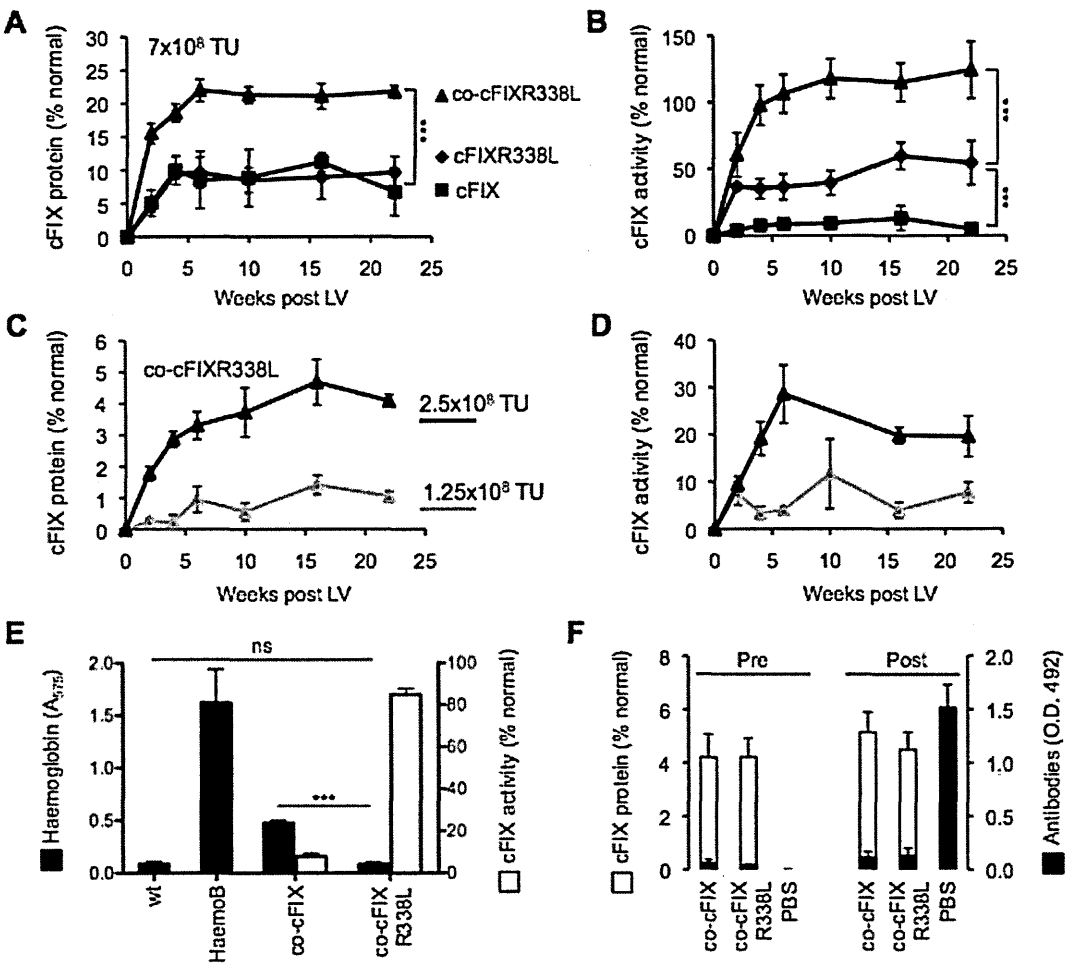


Figure 9: Codon-optimised cFIXR338L transgene in haemophilia B mice. cFIX expression and clotting activity measured by ELISA (A,C) and aPTT (B,D,E,F) respectively on plasma samples collected from treated mice at the indicated times after LV administration. VCN was measured at the end of the experiments in liver genomic DNA. Mice were treated with 7x10⁸ TU/mouse (A and B) of ET.cFIX.142T (squares, n=4; VCN 1.1±0.2) or ET.cFIXR338L.142T (diamonds, n=6; VCN 1.3±0.3) or ET.co-

cFIXR338L.142T (triangles, $n=7$; VCN 2.2 ± 0.2). Mice were treated with 2.5×10^8 TU/mouse (black line, $n=3$; VCN 1.4 ± 0.2) or 1.25×10^8 TU/mouse (grey line, $n=3$; VCN 0.6 ± 0.1) of *ET.co-cFIXR338L.142T*. Results are presented as mean \pm SEM. ***: $p_{\text{GLOBAL}} < 0.001$ (non parametric combination statistics). (E) Tail-clipping assay on haemophilia B mice treated with 2.5×10^8 TU/mouse of *ET.co-cFIX.142T* ($n=3$) or *ET.co-cFIXR338L* ($n=3$) as indicated. Blood loss (mean \pm SEM) was determined by measuring the absorbance at 575 nm of haemoglobin content in the saline solution in which the tail was placed (black bars, left axis); cFIX activity (mean \pm SEM; white bars, right axis). Wt ($n=5$) and untreated haemophilia B (HaemoB) mice ($n=5$) were used as controls. ***: $p < 0.001$, ns: not significant (ANOVA). (F) cFIX-specific antibodies (mean \pm SEM, black bars, right axis) and cFIX expression (mean \pm SEM, white bars, left axis) measured by ELISA in the plasma of mice treated with 5×10^8 TU/mouse of *ET.co-cFIX.142T* ($n=3$), *ET.co-cFIXR338L.142T* ($n=3$), or PBS ($n=3$) before or after immunisation with cFIX protein, as indicated.

To assess the possible risks associated with expression of hyper-functional FIX, we performed histopathological evaluation of liver, spleen, kidney, heart, lungs and brain of mice long-term reconstituted (10 months post LV) to supra-physiological FIX activity by co-cFIX-R338L LV (up to 125% of normal) and found no significant difference between the organs of mice treated with wt and hyper-functional transgene. Moreover, we determined D-dimer levels as a measure of fibrin degradation. D-dimers are not normally present in plasma, except when the coagulation system has been activated, as in the case of thrombosis²⁷⁰. We did not detect significant increase in D-dimer levels in mice treated with LV expressing hyper-functional R338L FIX transgenes, suggesting that thrombotic risk was not increased after gene therapy in haemophilic mice (not shown). However D-dimer levels are increased upon acute thrombotic events, thus repeating measures over time will be needed to more appropriately assess this risk. Moreover, thrombosis risk is expected to be low at the levels tested and further *ad hoc* studies in permissive thrombosis models are required to establish the long-term safety of delivering hyper-functional FIX transgenes²⁷¹. Our data, together with the known impact of the R338L mutation on substrate interaction rather than zymogen activation²⁶⁸, suggest that expressing limited amounts of hyper-functional FIX to reach a threshold therapeutic level represents a viable and promising strategy to improve the efficacy, feasibility and safety of haemophilia gene therapy.

These data prompted us to evaluate this hyper-functional co-cFIX-R338L transgene in the setting of LV-mediated liver gene therapy in the canine model. We thus produced a new large-scale batch of purified LV expressing the co-cFIX-R338L transgene

(2012/DG). Quality assessment of the vector preparations are reported in Table 5. This batch contained a total of 2.3×10^{10} TU, 3151 total μg p24, infectivity 7.3×10^3 TU/ng. While the infectivity of this LV preparation was slightly lower, total particles were approximately 3-fold higher and total TU approximately half as compared with the previous batch. Infusion of a haemophilia B dog with this preparation is expected at the time of this thesis being written.

Table 5. Quality controls of the large-scale purified LV Batch 2012/DG

Batch 2012/DG			Specification
Particles	μg p24/ml	13.7	For information only
Titre	TU/ml	1.0×10^8	$>1.0 \times 10^8$
Infectivity	TU/ng p24	7.3×10^3	$>1.0 \times 10^4$
Transgene Activity		Positive	Positive
Total DNA	$\mu\text{g}/\text{ml}$	0.4	For information only
Producer cell protein	ng/ml	210	For information only
Endotoxin	EU/ml	<0.5	<1
Sterility		Negative	Negative
RCL		Negative	Negative
Volume	ml	230	
Particles	Total μg p24	3151	
Titre	Total TU	2.3×10^{10}	
Endotoxin	Total EU	<115	

The table shows the results of selected quality control assays performed on the large-scale purified LV Batch 2012/DG. The specifications for GMP-grade LV production are listed. Please note that this batch is a research-grade production. EU: endotoxin units. TU: transducing units.

4.4 LVs pseudotyped with the baculovirus gp64 envelope protein mediated efficient liver gene therapy in haemophilia B mice

The VSV-G is widely used to pseudotype lentiviral vectors (LV), because it makes the vector pantropic and stabilizes vector particles, enabling the production of high-titre vector stocks and their long-term storage (see 1.4). However the use of alternative vector pseudotypes may be advantageous, when a more restricted tropism is preferable, and to overcome the limitations associated with VSV-G, such as toxicity in vector-producer cells and complement-mediated inactivation of the vector in serum^{225, 244, 264}.

For these reasons we evaluated the use of the baculovirus *Autographa californica* nuclear polyhedrosis virus gp64 envelope protein to pseudotype LV for liver gene therapy. This envelope protein was previously reported to confer tropism restriction against haematopoietic-lineage cells to pseudotyped LVs and improved resistance to complement-mediated inactivation²⁷². We first produced LVs pseudotyped with the gp64 envelope protein (gp64.LV) with a PGK.GFP cassette. We evaluated the extent of complement-mediated inactivation of gp64.LV in human and canine serum, as described in 4.1.1. The results show that gp64.LVs are more resistant to inactivation as compared to VSV-G-pseudotyped LVs (Figure 10A,B), with a maximum of 2-fold observed reduction in titre.

We next produced gp64.LV with a therapeutic cassette expressing hFIX driven by the ET promoter and including miR-142 target sequences (Figure 1). Haemophilia B mice were intravenously injected with 2.5 (n=2), 5 (n=4) or 10 (n=1) $\times 10^8$ TU/mouse and hFIX expression was measured on plasma collected at routine intervals after LV administration. The treatment resulted in all cases in stable levels of hFIX in the circulation and a vector dose-transgene expression direct correlation was observed (Figure 10C). None of these mice developed anti-hFIX antibodies (not shown). These data show that gp64.LV can be produced at high titres and mediate liver-directed hFIX expression at levels comparable to those obtained after matched doses of VSV-G-pseudotyped LVs in haemophilia B mice and may represent a valuable alternative to VSV-G for *in vivo* administration. However, VSV-G pseudotyped LV currently remain our preferred option due to the established experience with large-scale manufacturing and clinical usage.

Figure 10

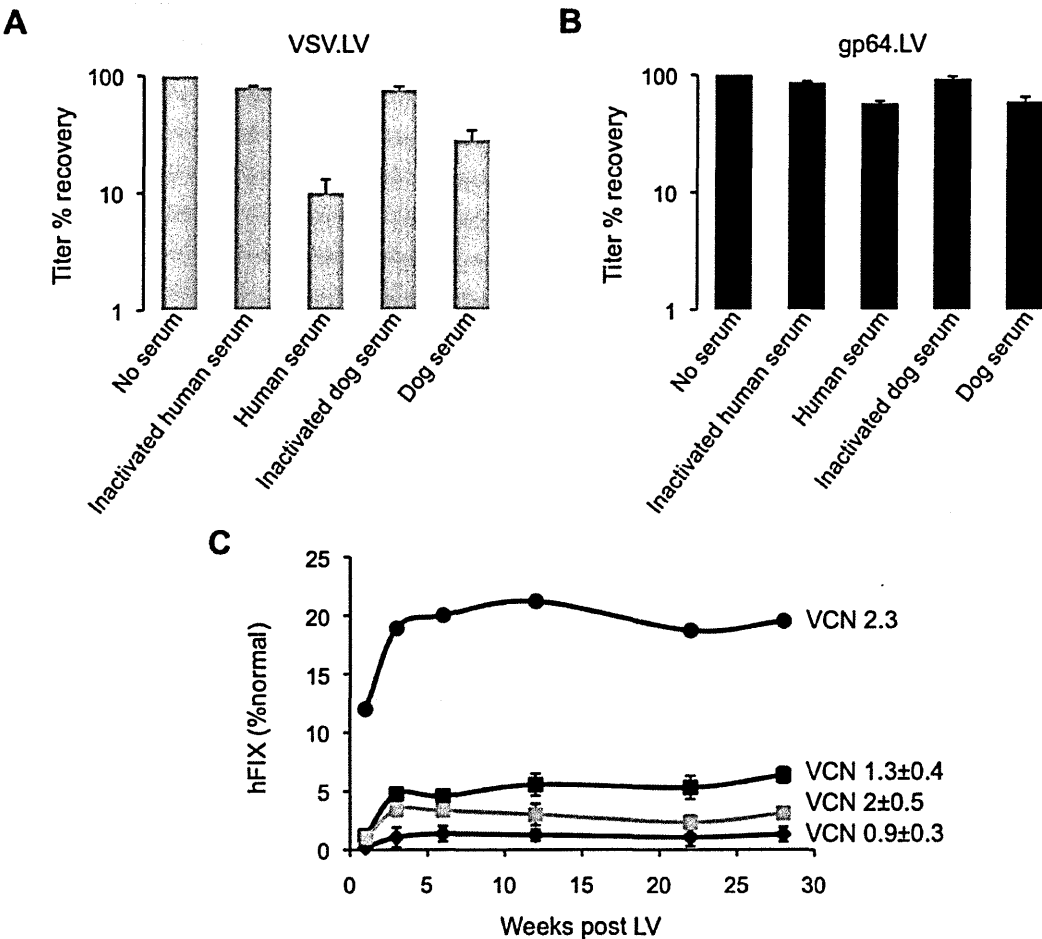


Figure 10: Gp64-pseudotyped LVs for liver gene therapy. Percentage of titre remaining after LV incubation with normal or heat-inactivated serum, as indicated, compared to no serum control, set as 100% recovery. PGK.GFP VSV-G-pseudotyped (A, grey bars) or gp64-pseudotyped (B, black bars) LVs were incubated for 1 h at 37° C with medium (no serum control) or serum of 2 healthy human donors, or a pool of canine serum. Results are presented as mean±range of 2 independent experiments. (C) hFIX expression measured by ELISA on plasma samples collected from treated mice at the indicated times after LV administration. Mice were treated with 5x10⁸ TU/mouse of VSV-G-pseudotyped (n=3, grey squares) or 2.5x10⁸ TU/mouse (n=2, black diamonds), 5x10⁸ TU/mouse (n=3, black squares), 10x10⁸ TU (n=1, black circles) of gp64-pseudotyped ET.hFIX.142T LVs. VCN in genomic DNA from the liver of treated mice measured at the end of experiment is indicated. Resultes are presented as mean±SEM (for n=3), mean±range (for n=2), or single values (for n=1).

4.5 LV biodistribution to KCs limits hepatocyte transduction to a different extent at different vector doses

We observed that there is no linear correlation between the injected LV doses and liver VCN, nor between transgene expression in hepatocytes and liver VCN (see for example Figure 6A,B, Figure 9C,D and Figure 10C). In order to clarify this aspect, we set out to examine the LV biodistribution among the liver cell subpopulations and to understand the relative contribution of these populations to the overall liver transduction. Specifically, we set out to determine VCN in different liver cell subpopulations after liver gene transfer at different LV doses and to correlate these data with the percentage of transgene-expressing hepatocytes. To this end, we first set up a method to separate cellular fractions enriched in hepatocytes (parenchymal cells, PCs) or non-hepatocyte cells (non-parenchymal cells, nPCs) from freshly dissociated livers and to further purify LSECs and KCs from total nPCs by FACS-sorting. We then intravenously administered 2.5, 5 or 10×10^8 TU/mouse ($n=10$ *per* dose) of LVs bearing the hepatocyte-specific ET.GFP.142T cassette to wt adult mice. At 2 months after LV administration we determined both the percentage of GFP⁺ hepatocytes in liver sections and VCN in whole liver DNA from half of the mice of each dose cohort ($n=5$). In some of the mice we extracted DNA from different liver lobes and found no difference in VCN between different lobes and between those and the VCN measured in whole liver (not shown). We perfused the other half of the mice ($n=5$) through the inferior vena cava with a collagenase solution. We centrifuge at low speed the digested liver tissue to obtain hepatocytes-containing pellets. We subject the supernatant to density gradient to further fractionate PCs and nPCs and FACS-sort the nPC-containing interface to obtain CD45⁻CD31⁺F4/80^{low} LSECs and CD45⁺CD11b⁺F4/80⁺ KCs (Figure 11), according to previously published protocols²⁷³. We then determined the VCN in these populations: fractionated liver PCs or nPCs, FACS-sorted LSECs or KCs.

Figure 11

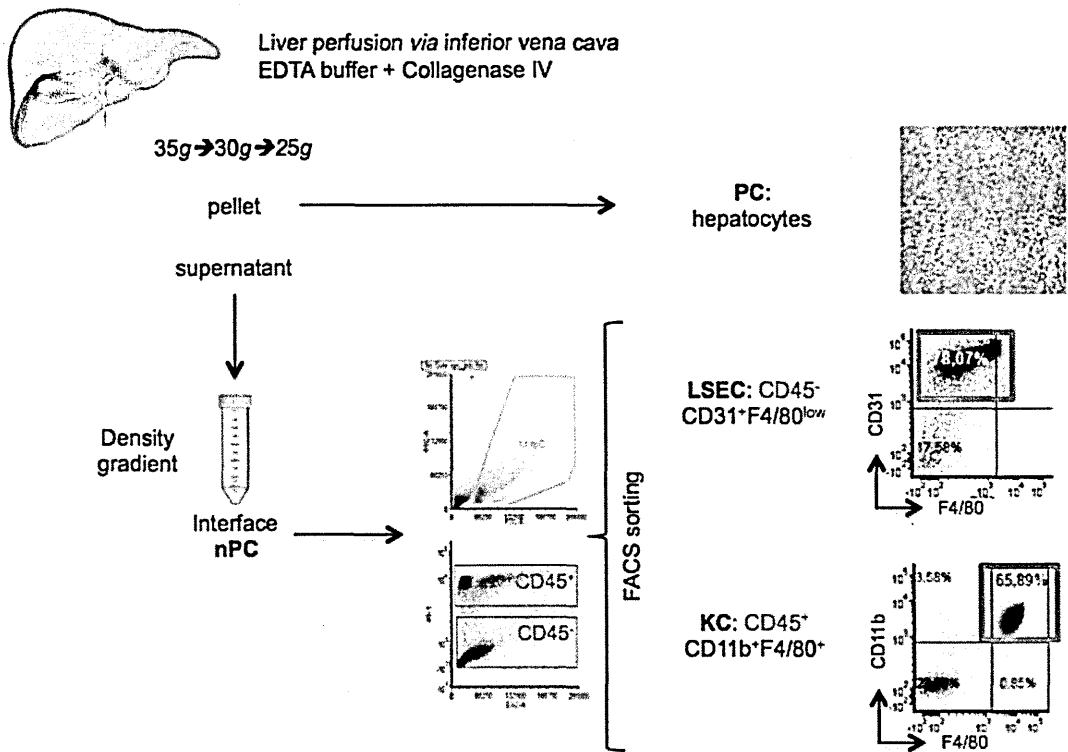


Figure 11: Schematic diagram of separation of PCs and nPCs from total liver. The liver is perfused through the inferior vena cava with an EDTA solution and collagenase IV. The digested tissue is centrifuged at low speed to obtain hepatocytes-containing pellets. The supernatant is subjected to density gradient and the nPC-containing interface is subsequently stained and FACS-sorted as indicated to obtain LSECs and KCs. Further details can be found in 3.15.

We first correlated the injected LV dose with the percentage of GFP⁺ hepatocytes or with the VCN measured in whole liver or in the cellular fractions enriched in PCs or nPCs (pre-sorting), or with the VCN measured in FACS-sorted LSECs or KCs (Figure 12A-F). The results show that KCs have a very high VCN (up to 27 LV copies *per cell*), even if they represent a relatively small proportion of the liver cells and that the VCN is generally higher in nPCs than in PCs, suggesting that a minority of the total LV particles approaching the liver can transduce the hepatocytes. Moreover, we found a non-linear correlation between the injected LV dose and the percentage of GFP⁺ hepatocytes or between LV dose and the VCN measured in the whole liver. In particular, while GFP⁺ hepatocytes and whole-liver VCN nearly doubled from 2.5 to

5×10^8 TU, there was a disproportionately higher increase in GFP+ hepatocytes and lower increase in VCN when further doubling the dose (Figure 12A,B). VCN in PCs also increased more than 2-fold when increasing the LV dose from 5 to 10×10^8 TU (Figure 12C). Instead there was a more linear correlation between LV doses and VCN in LSECs (Figure 12E). Interestingly, there was a strong increase in VCN both in fractionated pre-sorting nPCs and FACS-sorted KCs when increasing the LV dose from 2.5 to 5×10^8 TU and there was no further increase in VCN in both these cells population when doubling the LV dose from 5 to 10×10^8 TU (Figure 12D,F). These data suggest that increasing the LV dose between 2.5 and 5×10^8 TU results in a relatively modest increase in VCN in PCs and consequently transgene expression, while a large fraction of the LV particles ends up in nPCs, in particular KCs. It appears that LSECs transduction is minor. Instead, as the LV dose reaches a threshold between 5 and 10×10^8 TU, a “saturation” of LV transduction in KCs occurs and more LV particles are available to transduce hepatocytes and consequently the increase in VCN in PCs and transgene expression is more relevant. These effects are masked when the VCN is measured from the whole liver, because VCN changes in the opposite direction among PCs and nPCs and subtracts to each other.

According to published reports, we can consider the liver composed by 70% of PCs and 30% of nPCs, of which 50% are LSECs (15% of total liver cells), 20% are KCs (6% of total liver cells) and the remaining 30% are biliary ducts cells, hepatic stellate cells, infiltrating lymphocytes and other hematopoietic-lineage cells²⁷⁴. Thus, in order to gain a better understating of the LV biodistribution within the liver cell populations after i.v. injection of different doses, we calculated the relative contribution of the VCN for each sub-population, considered the theoretical relative abundance within the liver cells. In other words, we calculated VCN for each cell population “weighted” on their theoretical percentage of representation within the liver cells (e.g. VCN in PCs, VCN_{PC} , is multiplied by 0.7 to obtain the relative contribution of VCN_{PC} to total LV DNA in the liver, VCN_{NPC} is multiplied by 0.3 and so on). Based on this calculation, we found that at doses of 2.5 or 5×10^8 TU, approximately 30% of the LV DNA is found in PCs (and consequently about 70% is in nPCs), while at the high dose of 10^9 TU 57% of the LV DNA is found in PCs and the remaining 43% in nPCs. Among nPCs the vast majority of LV DNA is found in KCs (approximately 70% at 2.5 or 5×10^8 TU and 42% at 10^9

TU), while only 2-4% of LV DNA is found in LSECs at the doses tested. These data further support our hypothesis that a skewing occurs in the LV biodistribution between PCs and nPCs and better PCs transduction is achieved as the dose is raised above 5×10^8 TU.

Figure 12

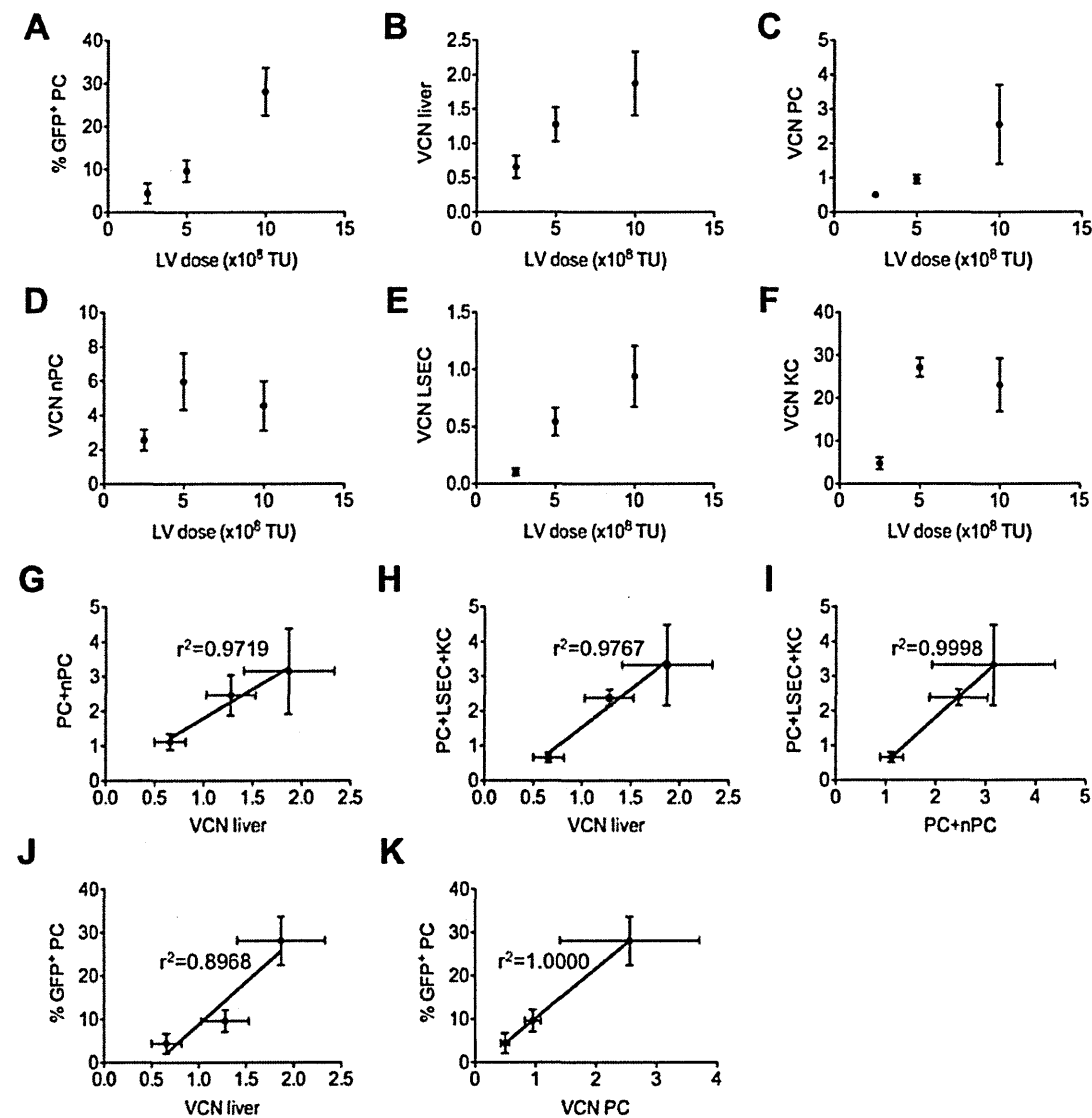


Figure 12: Biodistribution of LV within liver cell populations. Percentage of GFP⁺ PCs (A) in liver sections (5-10 optical fields scored from 6-8 non-consecutive sections/mouse), or VCN measured in genomic DNA extracted from whole liver (B), fractionated liver PCs (C) or nPCs (D), FACS-sorted LSECs (E) or KCs (F), as

described (Figure 11), of mice treated with the indicated dose (X axis) of ET.GFP.142T 2 months after LV administration (n=5). Correlations between VCN measured in whole liver (X axis) and liver VCN calculated based on the relative contributions of (G) VCN in fractionated PCs and nPCs, or (H) based on the relative contributions of VCN in fractionated PCs and FACS-sorted LSECs and KCs (Y axis). (I) Correlation of liver VCN calculated based on the relative contributions of VCN in fractionated PCs and total nPCs (X axis) and liver VCN calculated based on the relative contributions of fractionated PCs, FACS-sorted LSECs and KCs (Y axis). Correlations between the percentage of GFP⁺ PCs (Y axis) and (J) VCN in whole liver (X axis) or (K) VCN in fractionated PCs (X axis). Results are presented as mean±SEM.

We also correlated the measured VCN in whole liver DNA with that calculated summing either the relative contributions of the VCN in fractionated PCs and nPCs pre-sorting, or the relative contributions of the VCN in fractionated PCs and FACS-sorted LSECs and KCs (by the formula $VCN_{total} = (VCN_{PC} \times 0.7) + (VCN_{NPC} \times 0.3)$ or $(VCN_{PC} \times 0.7) + (VCN_{LSEC} \times 0.15) + (VCN_{KC} \times 0.06)$ respectively). We found a strong correlation between the observed whole liver VCN and the calculated total VCN based on the relative contributions of each cell subpopulations, suggesting that our calculation based on the theoretical relative abundance of the subpopulations within the liver is appropriate (Figure 12G,H). Note that our separation method only results in an enrichment in hepatocytes in the PCs fraction and we estimated a contamination by KCs between 1 and 2%, thus, since KCs have a high VCN, the measurement of VCN_{PC} results in a overestimation of the real VCN in hepatocytes. Therefore also the total VCN calculated as described before results in an overestimation with respect to the measured whole liver VCN, as it can be seen in Figure 12G,H. A very strong correlation and correspondence is found between the total VCN calculated summing the relative contributions of VCN_{PC} and VCN_{NPC} and that calculated summing the relative contributions of VC_{PC} , VCN_{LSEC} and VCN_{KC} (Figure 12I). We also correlated the percentage of GFP⁺ hepatocytes with the whole liver VCN (Figure 12J). We observed an exponential correlation consistent with the correlation between GFP⁺ hepatocytes and LV dose, as a 3-fold increase in GFP⁺ hepatocytes only corresponds to a modest increase in the VCN in the whole liver. Instead, a perfectly linear correlation ($r^2 = 1.0000$) is found between the GFP⁺ hepatocytes and the VCN_{PC} . This suggests that indeed the whole liver VCN does not correctly reflect the percentage of hepatocyte transduction, due to the different distribution of LV DNA observed within the liver cell subpopulations at different LV doses.

4.6 Proteasome inhibition decreases transduction of KCs and improves the efficiency of hepatocyte gene transfer by LVs *in vivo*

We set out to improve LV transduction of hepatocytes *in vivo* through a clinically viable strategy which did not cause depletion or toxicity on KCs. Proteasome inhibitors have been shown to possess anti-inflammatory properties and to decrease activation of KCs *in vivo*^{275, 276}. Moreover proteasome inhibition was reported to improve LV transduction in some cell types^{277, 278}, thus we reasoned that it may increase hepatocyte transduction as well. To this end, we assessed the impact of proteasome inhibition on LV biodistribution within the liver cell populations. We intravenously administered Bortezomib, a well-known and clinically used proteasome inhibitor, to mice prior to LV administration. Pharmacokinetic studies have shown that the peak of proteasome inhibition in the liver is achieved at 1 hour after i.v. administration of the drug²⁷⁹. We administered a single dose of Bortezomib (1 mg/kg) 1 hour before the administration of 5×10^8 TU/mouse of LVs bearing the hepatocyte-specific ET.GFP.142T cassette to normal adult mice (n=6). At 2 months after LV administration we determined both the percentage of GFP⁺ hepatocytes in liver sections and VCN in whole liver DNA from half of the mice (n=3). We determined the VCN in fractionated liver PCs or nPCs, FACS-sorted LSECs or KCs from the other half of the mice (n=3), as described in 4.5. We first compared the percentage of GFP⁺ hepatocytes observed in mice pre-treated with the drug with respect to that observed in mice treated with the same LV dose without Bortezomib pre-administration (Figure 13A). We found an almost 3-fold increase in transgene-expressing hepatocytes. We then compared the VCN in whole liver, VCN_{PC}, VCN_{NPC}, VCN_{LSEC} and VCN_{KC} observed in Bortezomib pre-treated mice with those observed in non pre-treated mice injected with the same LV dose (Figure 13B-F). We found no increase in whole liver VCN (Figure 13B). Interestingly we found an approximately 2-fold decrease in VCN_{NPC} and VCN_{KC} (Figure 13D,F). This finding may suggest that proteasome inhibition results in less KCs transduction at the time of injection or in death of the KCs with the highest VCN. Unexpectedly we found no increase in VCN_{PC} (Figure 13C), however, if we consider that the population enriched in PCs contains 1-2% of contaminating KCs and that VCN_{KC} is decreased by 2-fold, we

can estimate an approximately 1.5-fold increase in VCN_{PC} by subtracting 1-2% of VCN_{KC} by the respective VCN_{PC} .

Figure 13

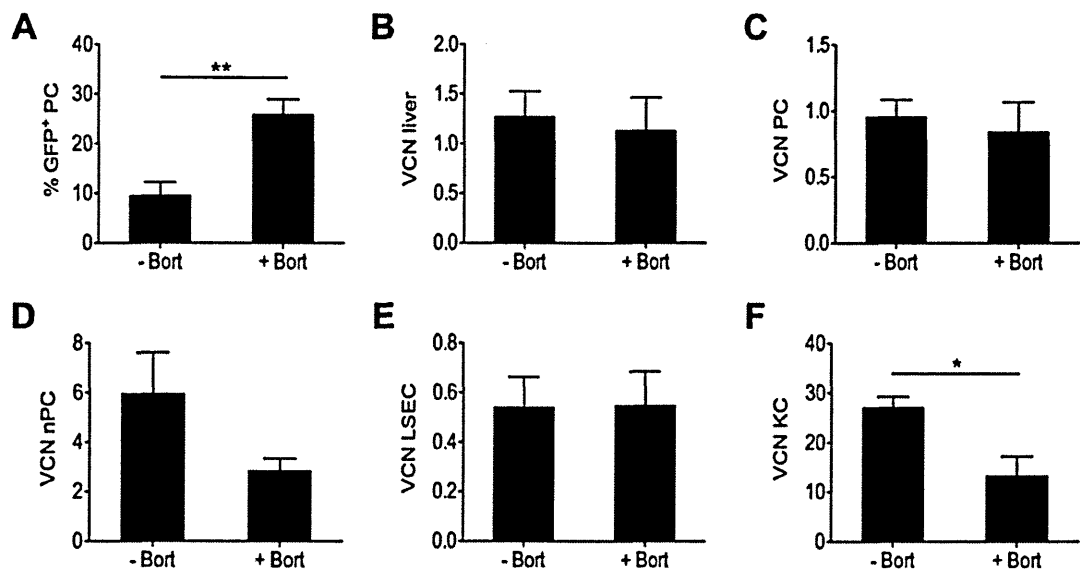


Figure 13: Biodistribution of LV within liver cell populations with or without pre-treatment with a proteasome inhibitor. Percentage of GFP⁺ PCs (A) in liver sections (5-10 optical fields scored from 6-8 non-consecutive sections/mouse), or VCN measured in genomic DNA extracted from whole liver (B), fractionated liver PCs (C) or nPCs (D), FACS-sorted LSECs (E) or KCs (F), as described (Figure 11), of mice treated with 5×10^8 TU/mouse of LV.ET.GFP.142T 2 months after LV administration, with (+ Bort, n=3) or without (- Bort, n=5) pre-treatment with Bortezomib (1mg/kg) 1 h before LV administration. Results are presented as mean±SEM. *: $p < 0.05$, **: $p < 0.01$ (t-test).

When we calculated the distribution of the LV DNA in the liver cell subpopulations, as explained in 4.5, we observed that 40% of LV DNA is found in PCs (and 60% in nPCs) in mice receiving Bortezomib prior to LV administration, compared to the 30% of LV DNA found in PCs (and 70% in nPCs) in mice not receiving the drug prior to LV administration. This change is likely to be underestimated for the reason described above. These data suggest that proteasome inhibition skews the LV biodistribution within the liver favouring transduction of PCs at the expense of KCs transduction.

4.6.1 Proteasome inhibition increases the potency of LV-mediated liver gene therapy in haemophilia B mice

To evaluate whether proteasome inhibition improves the efficacy of liver gene therapy for haemophilia B, we administered a single dose of Bortezomib (1mg/kg) to haemophilia B mice 1 hour before the injection of ET.hFIX.142T LVs. We measured hFIX levels in the plasma of treated mice at different time points after LV injection. As shown in figure 14A mice receiving the drug (n=4) consistently expressed 2- to 3-fold more hFIX as compared to mice which did not receive it (n=3). Mice were monitored for anti-hFIX antibodies and found to be negative, indicating that the treatment did not provoke a detectable immune response to the transgene. We determined VCN in gDNA extracted from the liver of treated mice 30 weeks after LV injection. There was no increase in the total vector content of the liver in bortezomib-pre treated mice, in line with data described before (Figure 13).

It is known that Bortezomib inhibits function and survival of pDC, which are major producers of type-I IFNs²⁸⁰. Since we have shown that type-I IFN signalling limits hepatocyte transduction²⁴¹, it is possible that Bortezomib down-regulates this pathway and consequently improves hepatocyte transduction. To test this hypothesis we evaluated whether Bortezomib pre-treatment increases or not hepatocyte transduction, upon i.v. injection of ET-hFIX.142T LVs in mice defective for type-I IFN receptor (*Ifnar1*^{-/-}; Figure 14B). We found that Bortezomib treatment strongly increases (approximately 5-fold) LV-mediated hFIX liver gene transfer in these mice, suggesting that the underlying mechanism cannot be ascribed to suppression of the IFN response.

To shed light on the mechanism by which proteasome inhibition increases LV hepatocyte transduction *in vivo*, we evaluated whether Bortezomib treatment increases LV transduction in primary hepatocytes *in vitro* as well. We transduced human primary hepatocytes with a low-dose PGK.GFP LV (MOI 2) in the presence or absence of Bortezomib (100 nM) given at the time of transduction. We calculated the percentage of GFP⁺ hepatocytes and measured VCN in transduced hepatocytes 1 week after transduction (Figure 14C-E). We observed a significant 2-fold increase in the percentage of GFP⁺ hepatocytes and an almost 2-fold increase in VCN when transduction was performed in combination with Bortezomib, as compared to those observed in hepatocytes transduced without Bortezomib. To exclude an effect of the

proteasome inhibitor on the half-life of GFP, Bortezomib was given 2 days after LV transduction and no increase in the percentage of GFP⁺ hepatocytes nor in VCN was observed in this condition. These data indicate that proteasome inhibition increases LV transduction of hepatocytes *in vitro* through a cell-autonomous mechanism (directly involving hepatocytes themselves).

Figure 14

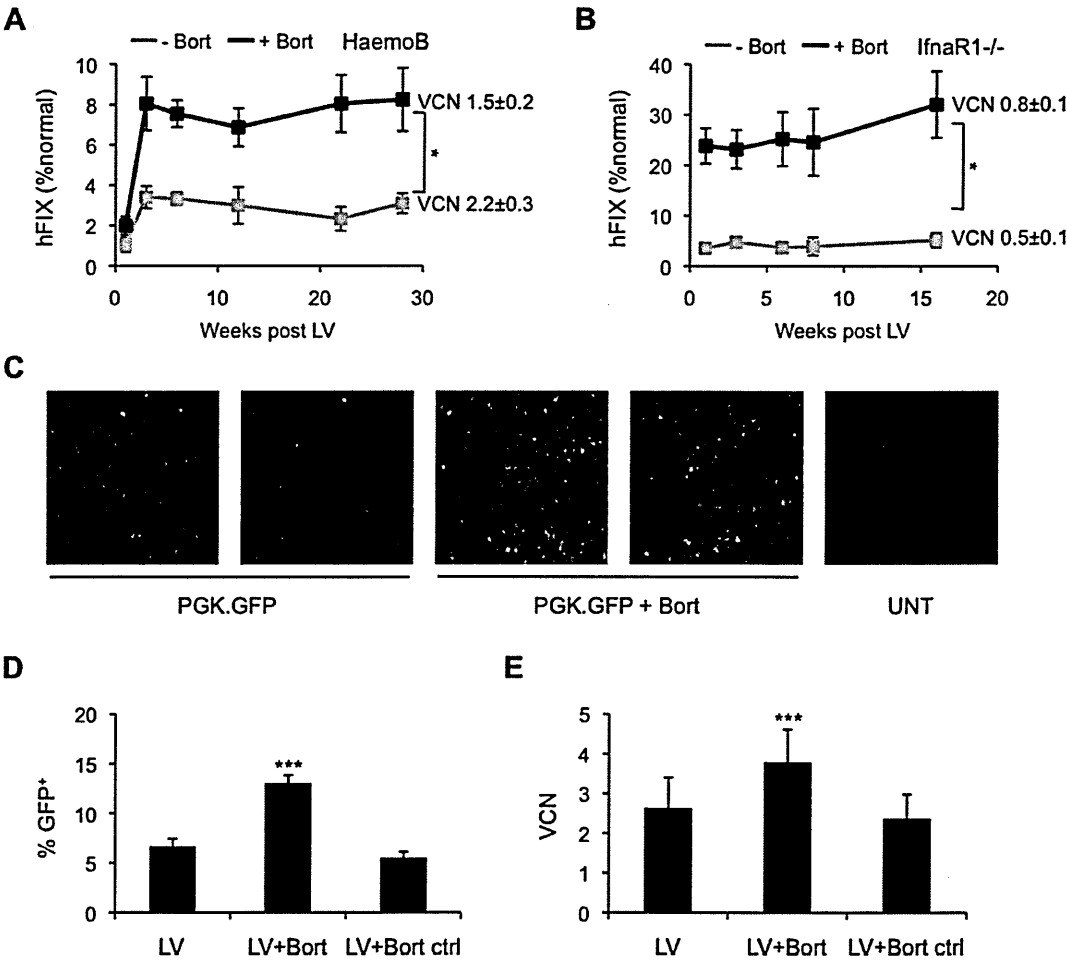


Figure 14: Proteasome inhibition in LV-mediated liver gene therapy. *hFIX* expression measured by ELISA on plasma samples collected from treated haemophilia B (A) or *IfnaR1*^{-/-} (B) mice at the indicated times after LV administration. Mice were treated with 5x10⁸ TU/mouse of ET.*hFIX*.142T LVs with (n=4, + Bort, black line) or without (n=3 HaemoB, n=4 *IfnaR1*^{-/-}, - Bort, grey line) pre-treatment with Bortezomib (1mg/kg) 1 h before LV administration. Results are presented as mean±SEM. (C) Representative images of human primary hepatocytes cultures transduced with

*PGK.GFP LVs (MOI 2), with (n=4) or without (n=4) Bortezomib (100 nM) at the time of transduction, or left UNT as indicated and analysed 7 days after transduction, by live fluorescence microscopy. Percentage of GFP⁺ hepatocytes (D; 7 fields/sample) and VCN (E) from the above described experiment. Nuclei were stained with Hoechst. To control for inhibition of GFP degradation Bortezomib (100 nM) was added 2 days after LV administration (n=3, LV+Bort ctrl). *: pGLOBAL<0.05 (non-parametric combination statistics); ***: p<0.001 (ANOVA).*

Overall our data show that a single-dose of proteasome inhibitor before LV administration improves hepatocyte transduction by 2- to 3-fold and this may represent a clinically viable strategy to improve the potency of LV for liver gene therapy.

4.7 LV-mediated liver gene therapy reverts anti-FIX antibodies and reconstitutes FIX expression in haemophilia B mice with pre-existing anti-FIX immunity

In order to evaluate whether LV-mediated liver gene therapy can eradicate pre-existing anti-FIX antibodies and establish FIX expression in haemophilia B mice, we performed gene therapy after inducing an anti-FIX immune response. We immunised adult haemophilia B mice with hFIX protein. All the immunised mice mounted a robust anti-hFIX humoral response and developed inhibitors at an average titre of 4 BU/ml with some mice having high-titre inhibitors (>10 BU/ml; Figure 15A-C). Four weeks later we divided the mice in 3 groups. One group received i.v. administration of 10⁹ TU/mouse of ET.FIX.142T LV (n=5), another group received i.v. administration of the same dose of LV expressing the unrelated antigen ovalbumin (ET.OVA.142T, n=3), while one group was vehicle-injected (PBS, n=4). We determined anti-FIX binding and neutralizing antibodies and FIX expression in the plasma of treated mice at routine intervals after immunisation (Figure 15A-D). In sharp contrast to PBS-treated mice, which maintained high-levels antibodies and even showed increased inhibitors titres (up to 6 BU/ml), all the mice receiving ET.FIX.142T LV showed a gradual decline in anti-FIX antibodies over the 8 weeks following gene therapy (12 weeks post immunisation) and inhibitors became undetectable (Figure 15A-C). Concomitantly with the decrease in anti-FIX antibodies, FIX expression gradually raised, reaching 25% of normal levels at 8 weeks after gene therapy, which is the expected level for the administered LV dose (Figure 15D). In contrast, mice receiving control ET.OVA.142T LV did not show any

decline in anti-FIX antibodies, which were found at levels undistinguishable from those found in saline-injected mice (Figure 15A,B).

Figure 15

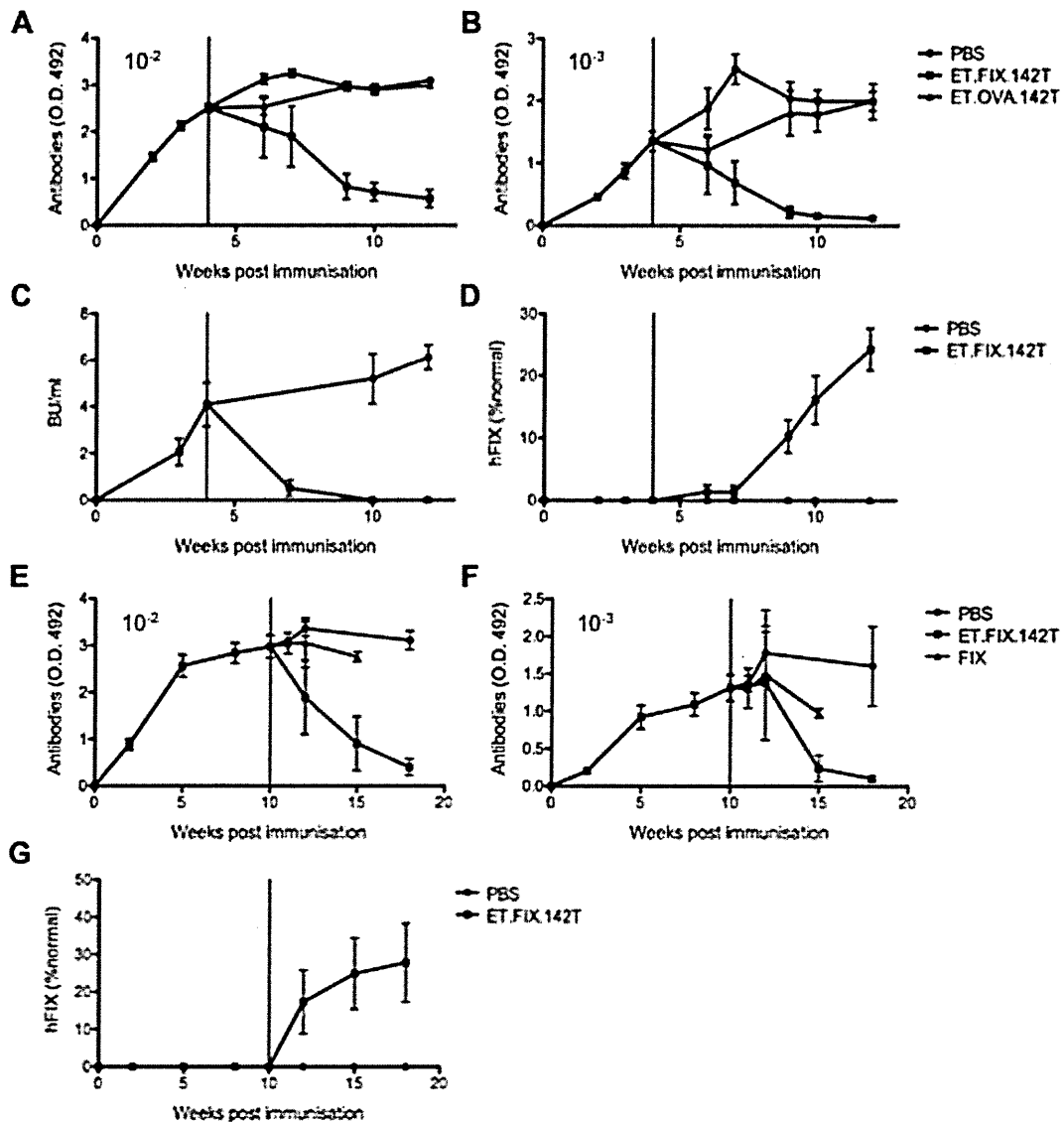


Figure 15: LV-mediated liver gene therapy in haemophilia B mice with pre-existing anti-FIX immunity. Anti-FIX total Ig antibodies (A,B,E,F) tested at 2 plasma dilutions, as indicated (10^{-2} A and E, 10^{-3} B and F), FIX expression (D,G) and anti-FIX inhibitors (C) measured in plasma samples collected from mice at the indicated times after immunisation. In A,B,C,D mice were immunised with hFIX protein in IFA and subsequently injected with PBS ($n=4$, circles) or 10^9 TU/mouse of ET.hFIX.142T ($n=5$, squares) or ET.OVA.142T ($n=3$, triangles) LVs 4 weeks after immunisation, as indicated. In E,F,G mice were immunised as above and subsequently injected with PBS

(n=3, circles), 10⁹ TU/mouse of ET.hFIX.142T (n=5, squares) LVs or with hFIX protein at 200-400 IU/kg i.v. every other day for 2 weeks (n=4, triangles) 10 weeks after immunisation, as indicated. Results are presented as mean±SEM.

We then repeated the experiment, this time performing gene therapy in haemophilia B mice at a later time after immunisation, when the anti-FIX humoral response was more mature. To this end we intravenously administered saline (PBS, n=3) or 10⁹ TU/mouse of ET.hFIX.142T LV (n=5) to haemophilia B mice 10 weeks after immunisation, when most of the antibodies-secreting B cells resided in bone marrow (A. Annoni, unpublished observation). We monitored anti-FIX antibodies and FIX expression in plasma of treated mice at routine intervals after immunisation (Figure 15E-G). Similarly to what was observed when gene therapy was performed 4 weeks after immunisation, LV-treated mice showed a gradual decline in anti-FIX antibodies over the 8 weeks following gene therapy (18 weeks post immunisation) and FIX expression was established at an average of 30% of normal levels, corresponding to the expected outcome of the administered LV dose (Figure 15G). In an attempt to compare the efficacy of gene therapy with that of ITI in reverting the anti-FIX antibody response, we administered to 4 haemophilia B mice an ITI-like regimen (consisting of high-dose recombinant hFIX protein at 10 IU/mouse corresponding to 200-400 IU/kg i.v. every other day for 2 weeks) 10 weeks after immunisation. Two out of 4 mice died during the course of this treatment (one of which within minutes after the third injection). We could not ascertain the cause of death, but it is possible that injection of the hFIX protein in these mice caused anaphylactic or immune complex-mediated hypersensitivity reactions²⁸¹. Follow-up of these mice was interrupted 5 weeks after the initiation of the ITI (15 weeks after immunisation) due the subsequent death of another of these 2 remaining mice. However, during this time ITI-treated mice did not show a reduction in anti-FIX antibodies (Figure 15E,F). These data indicate that liver gene therapy can revert established pre-existing anti-FIX binding antibodies and inhibitors and reconstitutes FIX expression to therapeutic levels in haemophilia B mice. These data also suggest that gene therapy may be more effective and safer than ITI in eradicating anti-FIX antibodies.

In order to shed light on the mechanism involved in the observed reduction of the anti-transgene antibody response after liver gene therapy, we studied the B-cell

response in treated mice. Gene therapy-treated mice showed significantly fewer anti-FIX antibody secreting B cells than saline-injected controls in the draining lymph node near the immunisation site at 10 weeks after LV administration (Figure 16A,B). Further analysis of other lymphoid organs such as the spleen and bone marrow will be required to better assess this response. We also found a strong reduction in the number of hFIX-specific memory B lymphocytes (B_{MEM}) in mice treated with gene therapy as compared to control saline-injected mice (Figure 16C,D). These data suggest that gene therapy resulted in a contraction of pre-existing transgene-specific B_{MEM} that in turn resulted in a decrease in anti-FIX antibodies producing B cells (and subsequent decrease in circulating antibodies) because of missing replacement at turnover. These data suggest that sustained endogenous production of hFIX upon liver gene therapy may cause anergy or apoptosis of B_{MEM} leading to a progressive inhibition of the antibody response, as reported for FVIII-specific B_{MEM} in a mouse model²⁸².

Figure 16

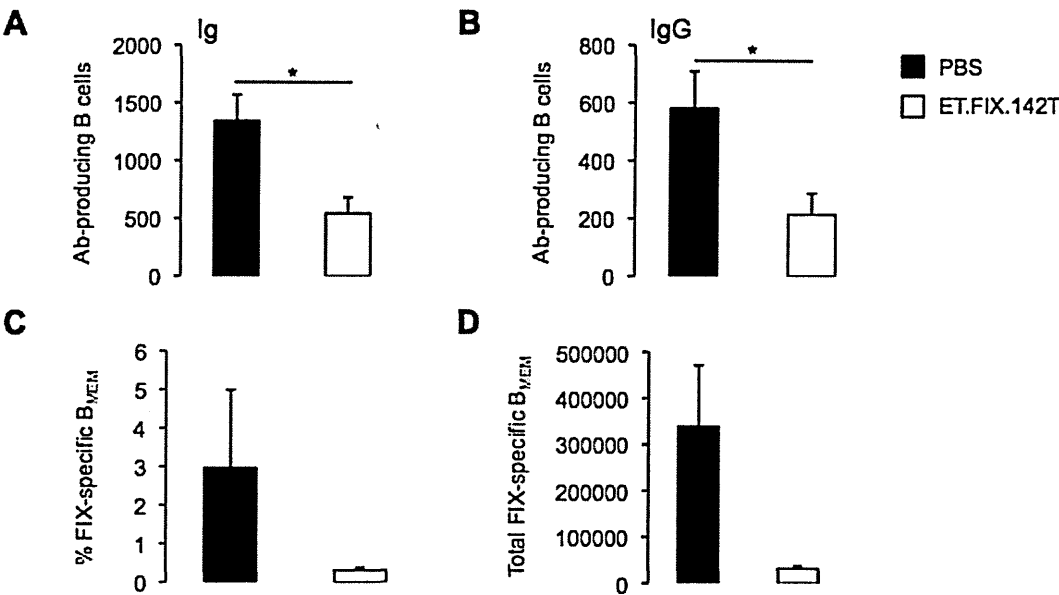


Figure 16: Analysis of B-lymphocyte responses after LV-mediated liver gene therapy in mice with pre-existing immunity. Anti-FIX total Ig (A) or IgG (B) antibody (Ab) producing B cells/ 10^6 , measured by ELISPOT in draining lymph nodes near the immunisation site of mice immunised with hFIX protein in IFA and subsequently treated with PBS ($n=4$, black bars) or 10^9 TU/mouse of ET.hFIX.142T LV ($n=5$, white bars) 4 weeks after immunisation (see Figure 15 A,B,C,D). Percentage (C) or total number (D)

of FIX-specific memory B lymphocytes (B_{MEM}) measured by flow cytometry in lymphnodes of mice immunised as above and treated with PBS ($n=3$, white bars) or 10^9 TU/mouse of ET.hFIX.142T LV ($n=4$, black bars) 10 weeks after immunisation (see Figure 15 E,F,G). FIX-specific B_{MEM} were identified as $CD19^+ B220^+ IgD^- IgM^- FIX-Alexa647^+$ cells. Results are presented as mean \pm SEM. *: $p<0.05$ (t-test).

4.8 Evaluating IDLVs for liver gene therapy

In order to assess whether integration is dispensable for long-term efficient liver gene replacement therapy, we set out to compare the efficiency of gene transfer, the levels and stability of transgene expression mediated by IDLVs and integrase-competent LVs (ICLVs) in a hepatocyte cell line, primary human hepatocytes and in the mouse liver, upon i.v. administration. We also set out to determine the nature of any persistent IDLV genome in the transduced livers. Moreover, we sought to investigate the ability of IDLV-mediated liver gene transfer to induce transgene-specific immune tolerance.

Towards these goals, we first generated GFP-expressing LV with either integrase defective or integrase competent packaging constructs. To drive transgene expression we used the hepatocyte-specific ET promoter. The vectors carried target sequences for miR-142 in the transgene 3' UTR (ET.GFP.142T, Figure 1). Physical particle content was determined p24 quantification. To determine infectivity, defined as TU *per* physical particle, we designed an *ad hoc* qPCR that selectively amplifies the reverse transcribed vector genome and discriminates it from plasmid DNA, carried over from the transfection used to produce the vectors (Figure 17). IDLV and ICLV had similar infectivity on several human cell lines (Table 6 and data not shown).

Figure 17

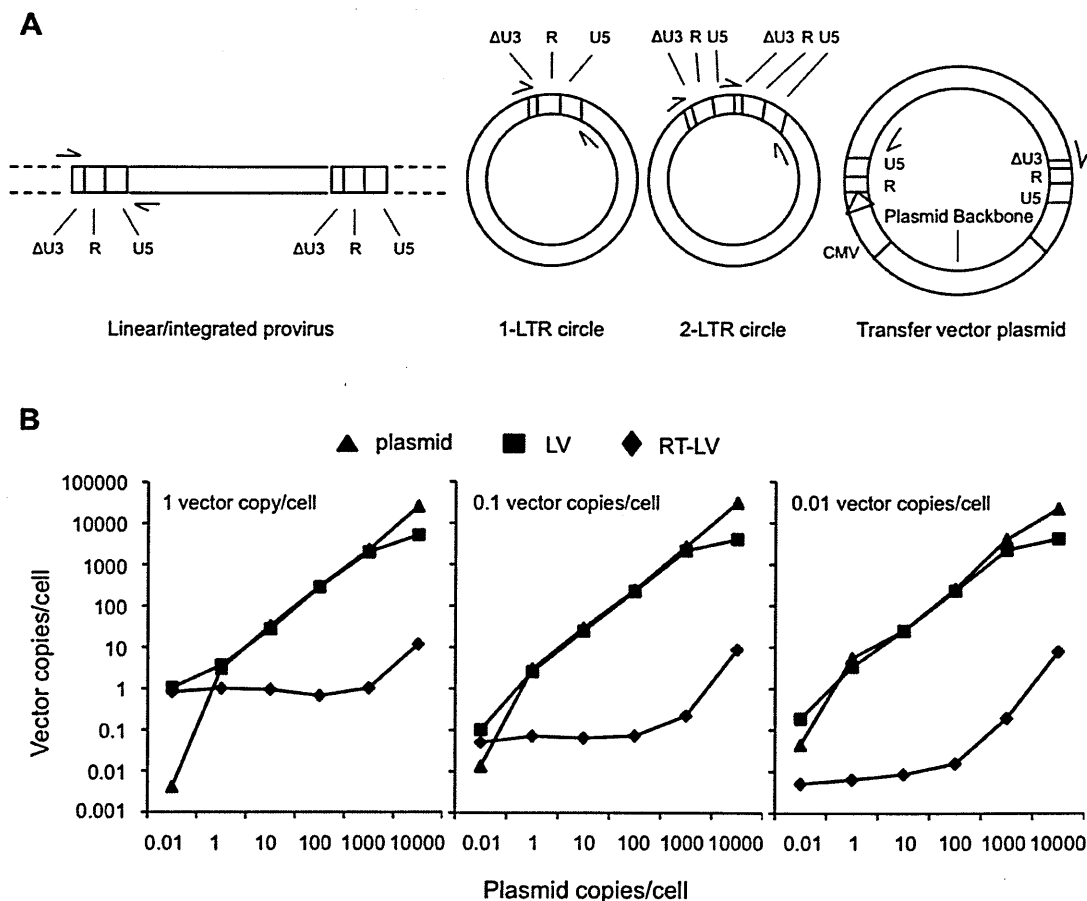


Figure 17: Quantification of reverse-transcribed vector genome in transduced cells. (A) Schematic representation of linear/integrated provirus, 1-LTR, 2-LTR circles and the transfer vector plasmid (used to produce the vector). The approximate position of the sense and antisense primers of the qPCR designed to quantify the reverse transcribed vector genomes is indicated (RT-LV). Note that PCR on transfer vector plasmid does not result in any amplification due to the missing DU3 region in the 5' LTR. (B) VCN measured in DNA extracted from standard cell clones with known vector content (previously determined by Southern blot) in which transfer vector plasmid is spiked in at different concentrations. VCN was determined using the qPCR designed to selectively amplify reverse transcribed genomes (RT-LV, diamonds, as shown in (A)), or using the standard qPCR assay used to amplify the lentiviral vector backbone (LV, squares), or using a qPCR assay designed to amplify the plasmid backbone (plasmid, triangles). Plasmid: plasmid sense 5'-gaatatacagagagtgaaggaactgttt-3', plasmid antisense 5'-tgtgaaatttgatgctattgctt-3', plasmid probe FAM 5'-tgcagcttataatggttacaa-3'. Note that while LV quantification is affected by transfer vector plasmid contamination and results in overestimation of VCN at increasingly higher spiked plasmid concentration, RT-LV quantification is not affected by transfer vector plasmid contamination and results in a reliable value, except at very high spiked plasmid concentrations.

Table 6. Titre and infectivity of IDLV vs ICLV

Vector	p24 (µg/ml)	Titre (TU/ml)	Infectivity (TU/ng p24)
IDLV n=3	211±9	3.7x10 ⁹ ±1.2x10 ⁹	1.8x10 ⁴ ±3.6x10 ³
ICLV n=3	162±28	5.0x10 ⁹ ±5.8x10 ⁸	3.9x10 ⁴ ±1.1x10 ⁴
<i>p</i>	ns (0.1270)	ns (0.3606)	ns (0.0907)

The table summarizes the p24, titre and infectivity of 3 different vector batches of ET.GFP.142T either packaged with an integrase-defective (IDLV) or integrase-competent (ICLV) configuration, as measured on 293T cells. Data are presented as mean±SEM. ns: not significant (t-test).

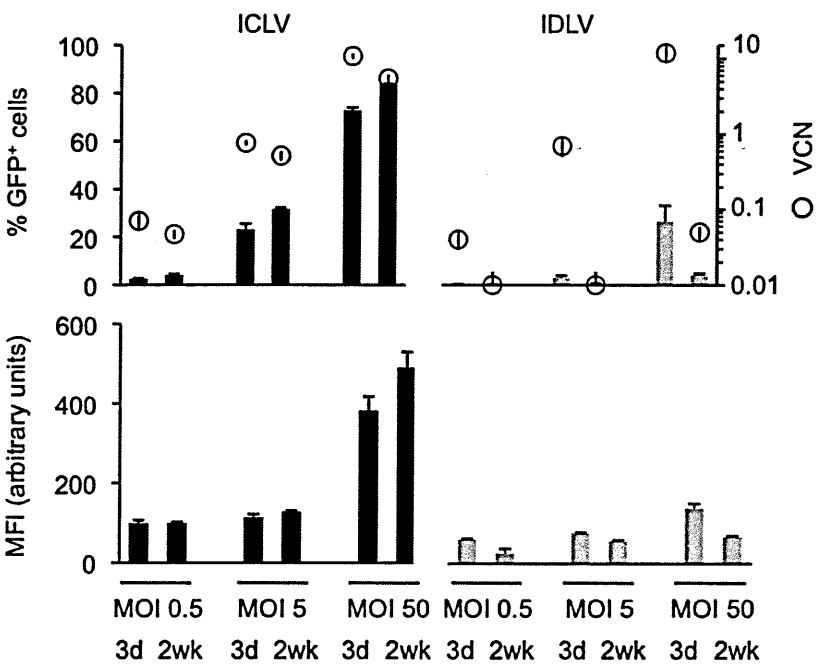
4.8.1 IDLV efficiently transfer episomal genomes into human hepatocytes *in vitro* but drive lower levels of transgene expression than their integration-competent counterparts

We compared IDLV and ICLV transduction efficiency and transgene expression levels in human cell lines and primary hepatocytes *in vitro*. We transduced the hepatocyte Huh7 cell line and measured VCN and GFP expression at 3 days and 2 weeks post-transduction (Figure 18A). Initially comparable amounts of reverse transcribed vectors were present in IDLV and ICLV-transduced cells. However, the frequency of GFP⁺ cells and the mean fluorescence intensity of GFP were lower in IDLV-transduced cells, compared to ICLV-transduced cells (p<0.01; n=3), indicating less efficient expression from the former vector. Analysis of the same cultures 2 weeks after transduction showed near complete loss of vector genomes and GFP⁺ cells in the IDLV-transduced cells, as expected for an episomal form.

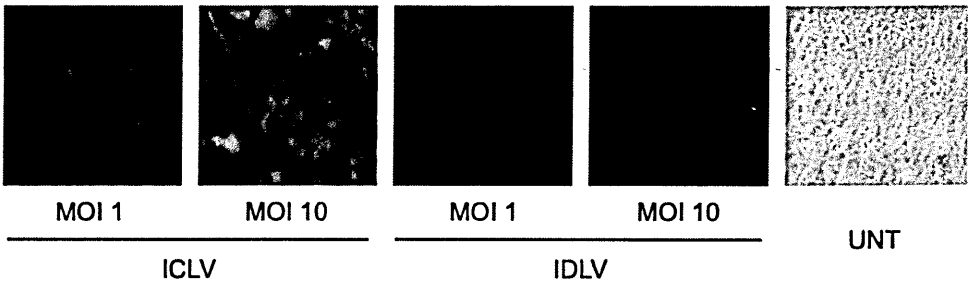
We then tested the same vectors on human primary hepatocytes, which do not proliferate in culture, and showed that IDLV attained similar levels of transduced vector genomes 1 week after transduction but expressed GFP at substantially lower levels than matched doses of ICLV (Figure 18B,C). Overall, these data indicate that IDLV efficiently transfer episomal vector genomes into hepatocytes. However, these forms provide a less proficient substrate for the transcriptional machinery than the integrated proviral vectors.

Figure 18

A



B



C

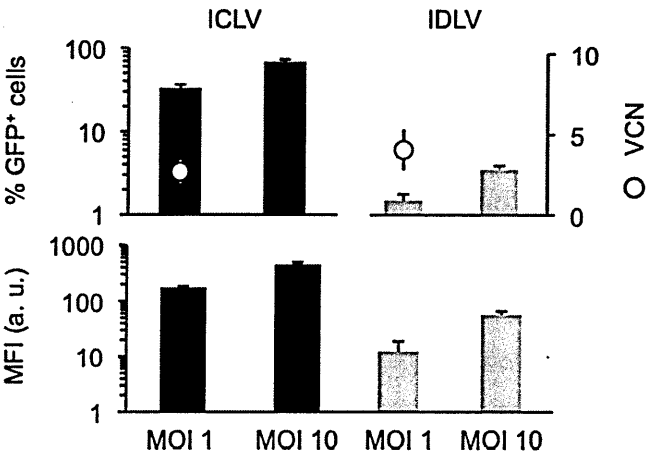


Figure 18: IDLV performance in hepatocytes cultures. (A) Percentage of GFP⁺ cells and MFI of GFP (left axis) and VCN (right axis) in Huh7 cells transduced with ICLV or IDLV at the indicated MOI and analyzed 3 days or 2 weeks after transduction by flow cytometry. Black bars correspond to ICLV-transduced cells, grey bars to IDLV-transduced cells. Circles show VCN. Results are presented as mean±SEM (n=3). (B) Representative images of human primary hepatocytes transduced as indicated or left UNT and analyzed by live fluorescence microscopy 1 week after transduction. Nuclei are stained with Hoechst. (C) Percentage of GFP⁺ cells and MFI of GFP (5 fields/sample; left axis) and VCN (circles, right axis) in cultures from quiescent human primary hepatocytes. Results are presented as mean±range (n=2).

4.8.2 IDLV support FIX expression from hepatocytes in mice

To ascertain that IDLV could be used to transduce hepatocytes *in vivo* we injected increasing doses (5, 20 and 40 µg p24, n=8) of GFP-expressing IDLV (ET.GFP.142T) intravenously in adult mice and measured GFP expression in hepatocytes and vector DNA content in the liver at 5 weeks post-injection (Figure 19A). A vector dose-dependent increase in hepatic transduction was apparent, yielding up to 13% of GFP⁺ hepatocytes in the treated livers, analyzed by GFP-specific immunostaining. Subsequently, we administered matched doses (20 µg p24) of GFP-expressing IDLV and ICLV (ET.GFP.142T) intravenously to adult mice (IDLV n=20, ICLV n=4, in 3 independent experiments) and measured GFP expression and vector DNA content in the liver at different times post injection (Figure 19B,C). At 1 week post injection, GFP-expressing hepatocytes were readily detectable in IDLV-treated mice, although to a substantially lower frequency and intensity as compared to those observed in mice treated with matched doses of ICLV. The content of reverse transcribed vector genomes was only slightly lower in mice treated with IDLV vs. ICLV and reached up to 1.5 vector copies *per* diploid genome. However, while GFP expression and vector content remain stable in ICLV-treated mice, the frequency of GFP⁺ hepatocytes and the vector content progressively diminished with increasingly longer times post-injection for the IDLV-treated groups.

Figure 19

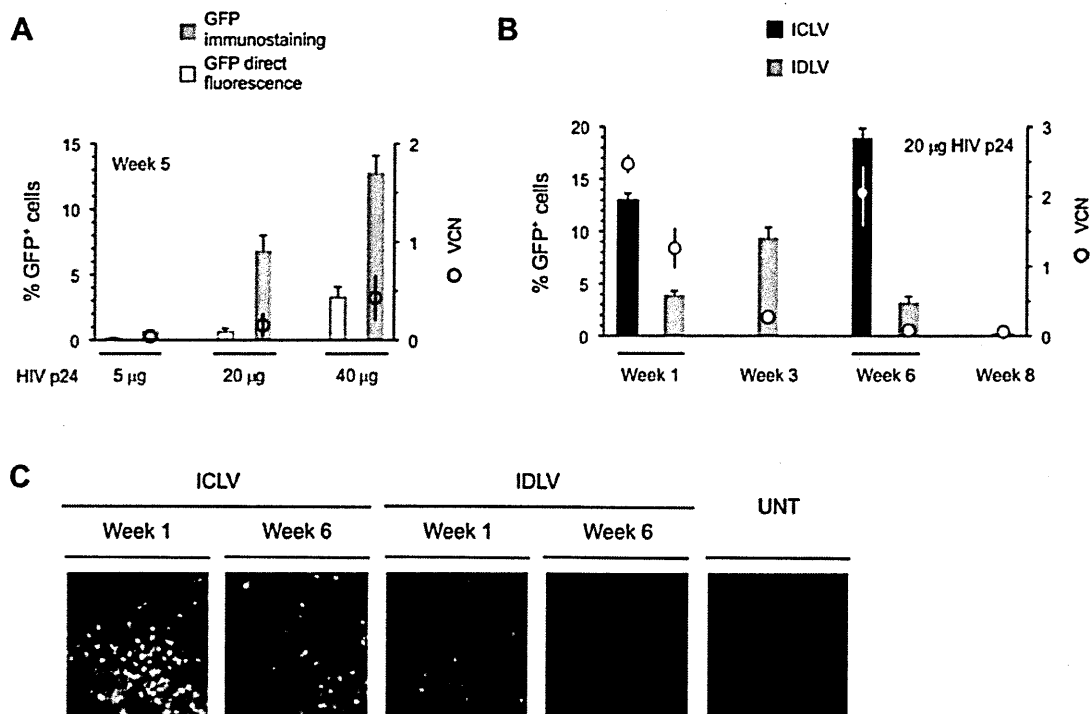


Figure 19: IDLV performance in the mouse liver. (A) Percentage of GFP⁺ hepatocytes and VCN in the liver of mice treated with the indicated p24 of IDLV.ET.GFP.142T and analyzed at the indicated time post-injection. GFP⁺ cells were identified either by immunostaining or direct fluorescence. Results are presented as mean \pm SEM of $n=3$ mice per time point, 5-10 optical fields scored from 5-10 non-consecutive GFP-immunostained (filled bars) or unstained (open bars) liver sections/mouse (left axis). Circles show VCN (mean \pm SEM, right axis). (B) Same analysis performed for IDLV- (grey bars) vs. ICLV- (black bars) treated mice, $n=3-7$ mice per IDLV time point from 3 independent experiments. (C) Representative images of unstained liver sections from mice reported in (B). Nuclei were stained with TOPRO-3.

We then administered matched high doses (260 μ g p24) of IDLV or ICLV expressing cFIX cDNA (ET.cFIX.142T) in adult haemophilia B mice (Figure 20, IDLV $n=15$, ICLV $n=8$). IDLV delivery resulted in prolonged production of FIX in mouse plasma at levels considered within the therapeutic range (up to 1.5% of normal levels). In agreement with the different efficiencies of expression observed for the GFP marker, the FIX levels supported by IDLV were up to 15-fold lower than those supported by the cognate ICLV. Twelve weeks post-injection, some of the IDLV-treated mice ($n=7$) and of the ICLV-treated ones ($n=3$) were subjected to 70% partial hepatectomy. In the

recovering mice, FIX levels remained comparable to those measured before hepatectomy in the ICLV-treated group (Figure 20A). In contrast, while FIX levels in the IDLV-treated mice were relatively stable prior to partial hepatectomy, they declined significantly shortly afterwards in the interval between week 12 and week 20 ($p < 0.05$; Figure 20B). Instead there was no statistically significant decline in FIX expression in the IDLV-treated cohort in the same time interval (week 12-20). Nevertheless, FIX expression had become sub-therapeutic after 1 year (Figure 20C).

Figure 20

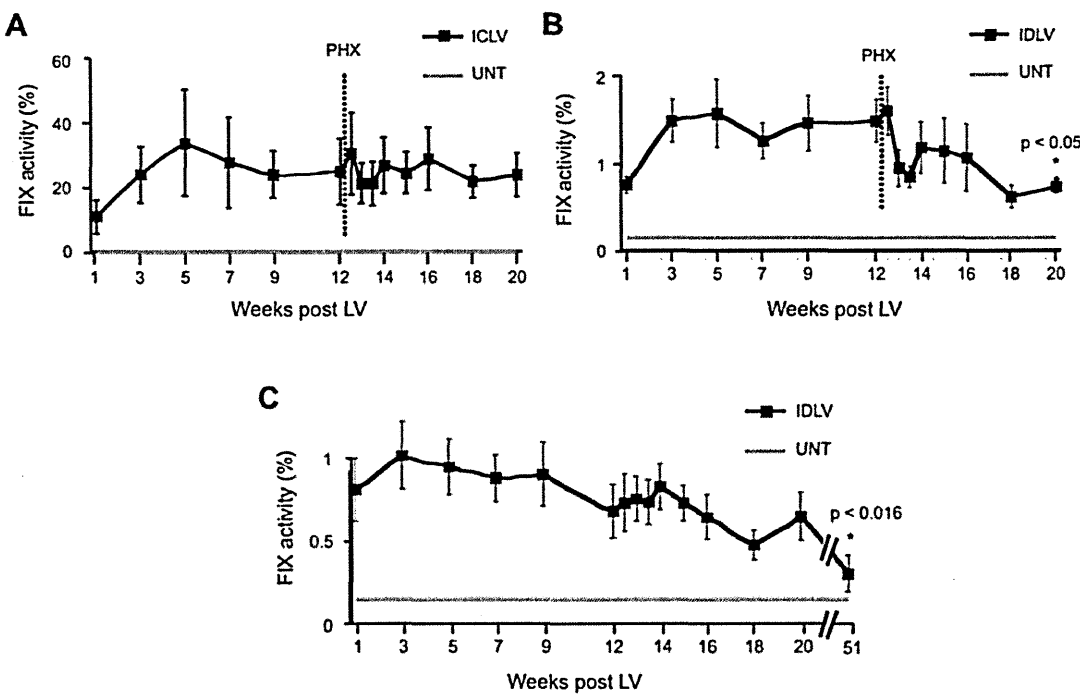


Figure 20: IDLV-mediated delivery of FIX in haemophilia B mice. FIX activity was measured on plasma samples collected from treated mice at the indicated times after LV administration. Mice were treated with 260 μ g p24 of ICLV.ET.cFIX.142T (A) or IDLV.ET.cFIX.142T (B). Partial hepatectomy (PHX) was performed at week 12. In both panels, grey lines correspond to untreated (UNT) mice. (C) Mice injected with IDLV.ET.cFIX.142T monitored for 1 year after LV administration and not subjected to partial hepatectomy.

These data indicate that IDLV, although being less efficient than their ICLV counterparts at expressing transgenes from human and murine hepatocytes, can support

therapeutically relevant levels of transgene expression *in vivo*. The post-hepatectomy drop in FIX observed in IDLV-treated mice suggests that FIX expression could be attributed mainly to non-integrated vector episomes. Thus, we investigated the residual IDLV integration frequency.

4.8.3 IDLV integrate only to background levels and with features incompatible with residual catalytic activity of the HIV integrase

As the D64V mutation reduces LV integration by 2 to 3 logs (see also Figure 18)²⁴⁵, low-level integration still occurs using this mutant. We therefore assessed residual IDLV integration in the treated livers. We retrieved vector sequences from the treated livers at 6 to 12 weeks after vector administration by highly-sensitive LAM-PCR^{258, 259} and deep sequencing. The number of unique IS found in the IDLV-treated liver samples was significantly lower as compared to the ICLV samples. In the IDLV liver samples (n=16) a total of 35 unique mappable integration sites (IS) could be recovered versus the 785 IS found in the ICLV samples ($p < 10^{-9}$), with an almost 100-fold lower frequency of retrieval (Table 7). Accordingly, we retrieved an excess 2-LTR junctions in IDLV-treated liver samples as compared to ICLV-treated ones, suggestive of the presence of episomal vector forms (2-LTR junctions: IDLV: 54.5%, ICLV: 2.8%; Table 7 and Figure 21). The few retrieved integrations did not show an enrichment for genes and frequently showed deletion in the LTR ends (IDLV: 25,7%, ICLV: 0,6%; Table 7).

These studies indicate that the occurrence of background integration in treated livers exhibits molecular features reminiscent of those described for plasmids and other types of episomal DNA. This analysis further underscores the minimal risk of insertional mutagenesis by IDLV.

Table 7. Deep sequencing of ICLV- and IDLV-transduced liver samples

Vector	Raw Sequence Reads	1-LTR/ Internal Control	2-LTR	Reads with LTR+ ≥20nt Genomic Sequence	Unique Mappable Integration Sites	LTR Deleted at Genome Junction by >3nt
ICLV						
	2787	211	51	1688	152	2 (10,11 nt)
	2179	13	0	244	244	1 (22 nt)
	3169	896	53	101	101	
	2822	250	258	288	288	2 (8,23 nt)
IDLV						
	1451	284	760	340	0	
	971	530	237	186	2	
	1227	373	82	767	0	
	1365	466	154	26	2	1 (21 nt)
	2264	371	838	841	2	
	2752	400	996	1322	1	
	1349	221	795	308	4	
	1173	193	587	277	3	1 (11 nt)
	2809	347	1538	502	1	1 (8 nt)
	1722	215	801	421	4	2 (19,24 nt)
	3494	350	2124	916	4	2 (14,21 nt)
	3260	223	2397	500	2	
	3291	316	1799	621	4	1 (25 nt)
	2771	330	1963	232	2	
	2882	275	2795	318	0	
	3251	241	1787	808	4	1 (8 nt)

The table shows 454 sequencing results of ICLV- and IDLV-transduced liver samples. 1-LTR and vector internal control fragments (resulting from the 3’LTR-U3 LAM-PCR primer annealing at the 5’LTR-U3) cannot be distinguished by sequencing. Of note, the 2-LTR LAM-PCR product (restriction enzyme Tsp509I, AATT) exhibits only a length of 18 bp without LTR and linker sequence, resulting in an overrepresentation of sequenced 2-LTR amplicons versus 1-LTR and Internal Control (see Figure 21). 1-LTR: 1-LTR amplicon. 2-LTR: 2-LTR amplicon. nt: nucleotides.

Figure 21

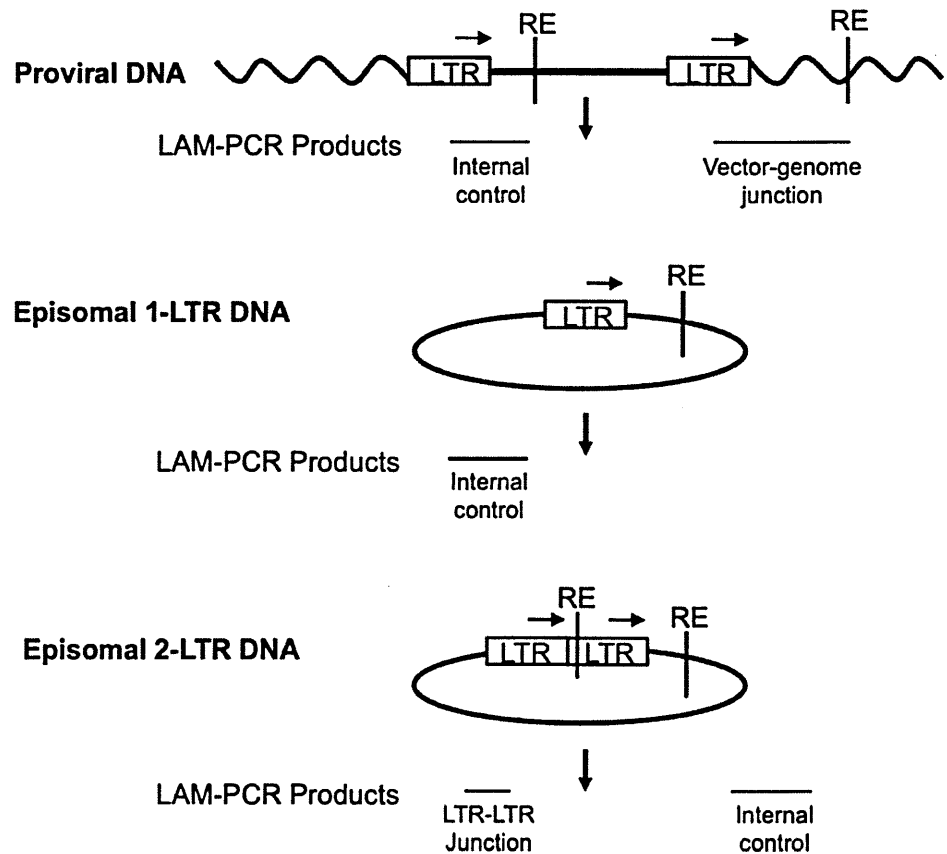


Figure 21: Schematic overview of proviral and episomal IDLV-based 3'LTR LAM-PCR results. The 3'LTR LAM-PCR primer sets (arrows) are hybridizing to the unique 5' region present in both 5' and 3' LTR sequences. In case of integrated proviral DNA, LAM-PCR results confer either to the vector-genome junction or to internal vector sequences, termed "internal control". In case of episomal 1-LTR circles, only an internal control can be detected. In case of episomal 2-LTR circles, an internal control and the LTR-LTR junction can be identified. Of note, the use of one restriction enzyme (RE) generates internal control and LTR-LTR junction LAM-PCR products of defined lengths, respectively (Table 7).

4.8.4 IDLV delivery tolerises the recipient to foreign antigens and induces antigen-specific Tregs

We then investigated whether hepatocyte-targeted expression by IDLV induces transgene-specific immunological tolerance. To investigate whether active tolerance was established, we challenged treated mice by i.m. vaccination with GFP-encoding plasmids and evaluated the frequency of GFP-specific CD8⁺ T cells in spleen and liver

(Figure 22A,B). Whereas the low frequency of GFP-specific CD8⁺ T cells induced in the spleen and liver of IDLV.142T-treated mice did not increase upon re-challenge, there were many more antigen-specific effectors in the control unregulated IDLV-treated mice after the primary (IDLV) and the secondary challenge (plasmid i.m.). In addition, residual levels of vector genomes were still detectable in the liver of mice previously treated with IDLV.142T after re-challenge (Figure 22B).

Figure 22

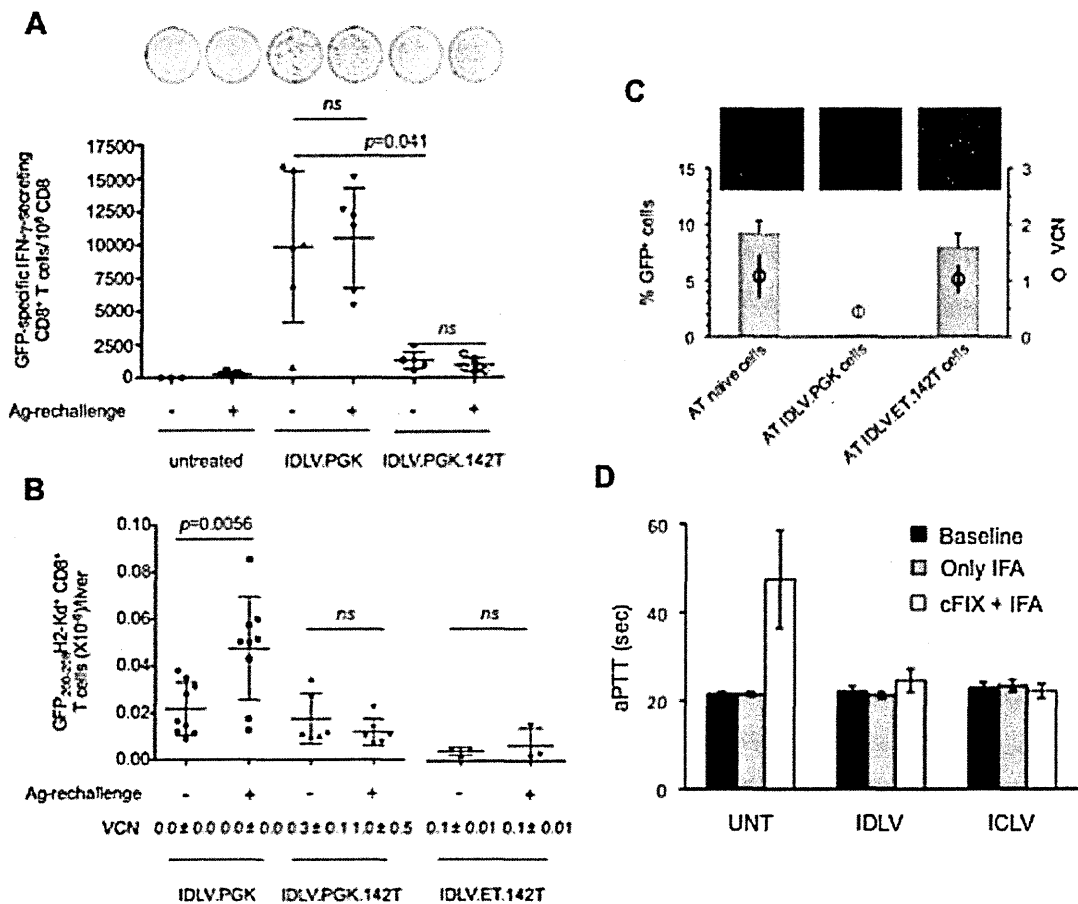


Figure 22: Transgene-specific tolerance after IDLV liver gene transfer. (A) Number of IFN- γ -producing GFP-specific CD8⁺ T cells per 10⁶ total CD8⁺ T cells in the spleen of mice subjected or not to antigen re-challenge (by i.m. vaccination with GFP-encoding plasmids) 6 weeks after IDLV treatment. Single values are plotted and the mean \pm standard deviation (SD) is shown (n=6/group). Representative wells are shown on top. (B) Number of the CD8⁺ GFP₂₀₀₋₂₀₈-pentamer-positive T cells infiltrating the liver of mice treated with the indicated IDLV (IDLV.PGK n=9; IDLV.PGK.142T n=6; IDLV.ET.142T n=3) after antigen re-challenge. Single values are plotted and mean \pm SD

is shown. Mean \pm SD VCN in the liver is indicated. (C) Percentage of GFP⁺ hepatocytes and VCN in the liver of immune-deficient mice previously injected with ET.GFP.142T ICLV and adoptively transferred (AT) with cells derived from naïve, IDLV.PGK- and IDLV.ET.142T-treated mice (n=3/group). Mean \pm SEM percentage of GFP⁺ hepatocytes (left axis). Circles show VCN (mean \pm SEM, right axis). A representative field is shown on top. Note that residual vector DNA in the liver of mice in which GFP-expressing hepatocytes were completely cleared can be ascribed to the persistence of vector genomes in transduced macrophages and endothelial cells that did not express GFP. (D) Plasma samples from the various groups of animals were screened for anti-FIX neutralizing antibodies using an aPTT mixing assay using a positive control of 2.6 BU and a known negative control. Samples were assayed in a blinded fashion. Groups include haemophilia B mice treated with the indicated ET.cFIX.142T vector or untreated (UNT) 12 weeks after vector administration, with or without challenge with cFIX and IFA.

To further confirm that IDLV.142T is able to direct the immune system towards immune tolerance induction to transgene antigens, we reconstituted immune-deficient recipients by adoptive transfer of immune cells derived from either immunized (IDLV-treated) or tolerised (IDLV.142T-treated) mice (Figure 22C). Rag2^{-/-}γ-chain^{-/-} mice were therefore first transduced with hepatocyte-targeted ICLV (ET.GFP.142T) to obtain robust GFP expression in hepatocytes. A pool of splenocytes and liver lymphocytes derived from naïve, control IDLV-treated or IDLV.142T-treated animals were transferred 1 month later. While comparable amounts of GFP-expressing hepatocytes were detected in the mice reconstituted with naïve cells or cells derived from tolerant (IDLV.142T-treated) mice, a complete clearance of GFP⁺ hepatocytes was observed in recipient mice reconstituted with cells derived from control unregulated IDLV-treated mice, accompanied by loss of vector DNA. These results indicate that only those cells derived from control mice exhibited full effector potential upon adoptive transfer, while the effector cells derived from tolerised mice (IDLV.142T-treated) are kept in check by the induced regulatory compartment. Taken together, these data indicate that GFP-specific immunological tolerance was established.

To assess if immune tolerance could be induced by IDLV towards a therapeutically relevant secreted antigen, we evaluated the anti-FIX immune response in haemophilic mice treated with FIX-encoding IDLV.142T. Bethesda assays showed no anti-FIX inhibitory activity (Figure 22D). Importantly, when these IDLV.142T-treated mice were challenged with FIX protein in IFA they remained negative for anti-FIX neutralizing antibodies. In contrast, haemophilic mice that were not injected with any vector but

immunized with FIX and IFA showed induction of neutralizing antibodies (Bethesda titer > 2.6 BU vs. below the level of detection in all recipient mice in the other cohorts). These results indicate that FIX-specific immunological tolerance was established.

To define whether antigen specificity of liver infiltrating Tregs is affected by IDLV.142T administration, antigen-driven conversion of naïve CD4⁺FOXP3⁻ T cells into FOXP3⁺ Tregs was evaluated. To this end, we adoptively transferred naïve CD4⁺ T cells obtained from double transgenic mice for an OVA-specific TCR (MHC II restricted, OT-II) and a GFP reporter knock-in downstream to the *Foxp3* promoter. OT-II CD4⁺FOXP3⁻GFP Ly5.2 T cells were isolated, FACS-sorted to deplete FOXP3⁺ cells, and adoptively transferred into naïve Ly5.1 recipient mice before the injection of OVA-encoding IDLV or IDLV.142T (Figure 23A). Three weeks later recipient mice were euthanized and GFP expression, used as surrogate marker for FOXP3, was determined in OT-II Ly5.2 CD4⁺ T cells. A significantly higher frequency of OVA-specific Tregs was detected specifically in the IDLV.142T-treated mice as compared to the untreated or control unregulated IDLV-treated mice (Figure 23B). These results indicate that the pattern of OVA expression driven by miR142-regulated IDLV in the liver favoured the conversion of transgene-specific naïve CD4⁺ T cells into transgene specific FOXP3⁺ induced Tregs (Figure 23B,C).

Figure 23

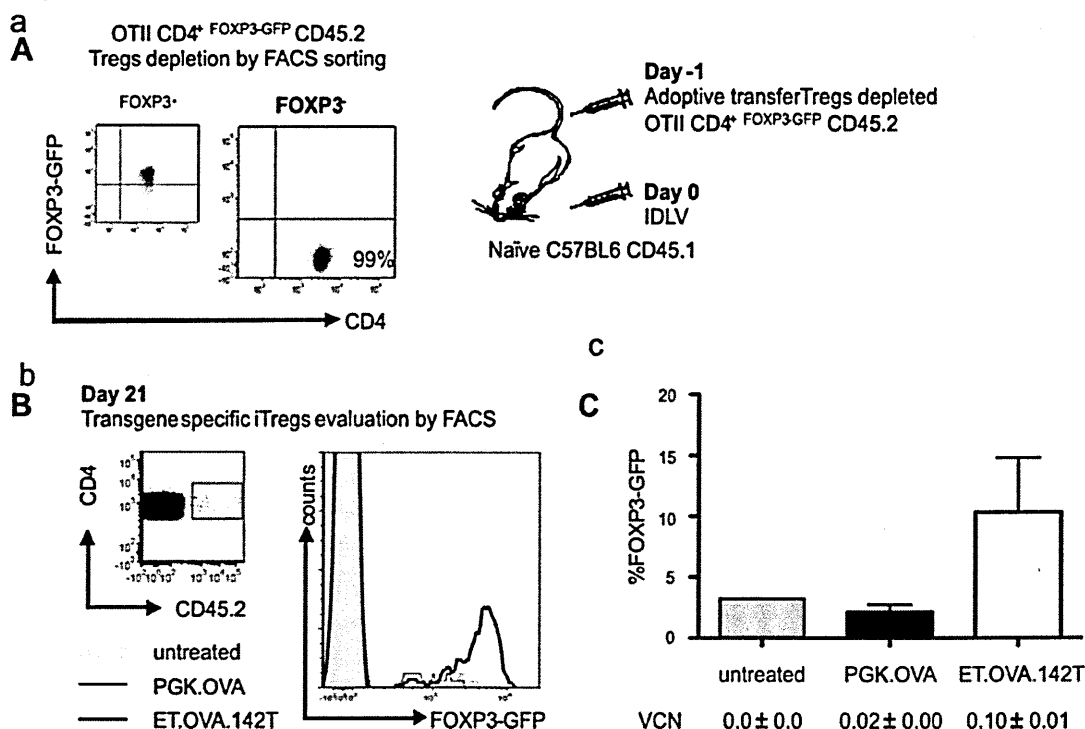


Figure 23: Induction of transgene-specific Tregs by IDLV treatment. CD4⁺ cells isolated from OTII Ly5.2 (CD45.2) Foxp3-GFP transgenic mice were FACS-sorted to remove GFP⁺ cells obtaining a homogeneous population of CD4⁺ non-regulatory T cells with a unique antigen specificity (OVA₃₂₃₋₃₃₉ presented in IAb molecule). (A) Tregs-depleted OTII CD4⁺GFP⁻ cells (2.5x10⁶/mouse) were adoptively transferred intravenously into naïve C57BL/6 Ly5.1 (CD45.1) recipient mice one day before the injection of IDLV.PGK.OVA (n=3) or IDLV.ET.OVA.142T (n=3). Three weeks after IDLV administration livers were harvested and infiltrating lymphocytes isolated. OVA-specific induced Tregs were measured as GFP⁺ cells gated on CD4⁺Ly5.2⁺. (B) A representative histogram and (C) mean±SEM percentage of induced Tregs are shown.

Overall, these data indicate that hepatocyte-targeted expression of foreign antigens including intracellular or secreted, therapeutically relevant (FIX) and model antigens (GFP, OVA) by IDLV can result in a state of transgene-specific immunological tolerance, due to induction of transgene-specific regulatory compartment. The mechanisms underlying the observed outcome are likely to be different between intracellular and secreted antigens and will deserve further studies.

5.DISCUSSION

5.1 LV-mediated liver gene therapy in haemophilia B dogs

The progression from small to large animal models has established a paradigm that is often used before the initiation of human trials, as it allows investigators to address issues of scaling up vector production to access larger amount of target tissue, to determine the toxicity and immune response to the procedure, the duration of transgene expression and the degree of correction of the disease phenotype in a mixed genetic or outbred animal²⁸³. The dog has been widely announced for its utility as a model for human hereditary diseases such as the haemophilias, lysosomal storage disorders and myopathies²⁸⁴. NHPs have also been largely adopted to perform pre-clinical studies and predict the toxicity of viral vectors administrations as well as the efficiency of expression cassettes with human transgenes^{172, 175, 207}. However only few disease phenotypes are conveniently available in these animals and the restrictions to lentivirus infections in most of them may limit their usefulness in evaluating LV-mediated gene therapies²⁸⁵.

We have also shown that canine hepatocytes can be efficiently transduce *in vitro*, indicating that there are no major obstacle in translating LV-mediated liver gene therapies in these animals. Since little is known about microRNAs in dogs²⁸⁶, we also assessed the expression levels of some microRNAs and activity of miR-142 in a canine hematopoietic-lineage cell line and verified the applicability of miR-142-regulated LVs for liver gene therapy or potentially other therapeutic strategies³⁶.

Taking advantage of a manufacturing process previously validated for the production of clinical-grade LVs for *ex vivo* gene therapy, we were able to produce large-scale batches of purified LVs qualified for *in vivo* administration and have performed the "first-in-dog" liver-directed LV gene therapy to our knowledge. Our results obtained in haemophilia B mice indicate that the "effective" LV dose to achieve 5-10% of normal FIX levels (and convert a severe to a mild haemophilic phenotype) corresponds to 5×10^8 TU/mouse ($2\text{--}2.5 \times 10^{10}$ TU/kg), equivalent to 10-20 μg p24/mouse (0.5-1 mg p24/kg) considering a standard particle infectivity of 2.5-5 TU/ng p24. According to manufacturing capability and precautions dictated by first testing in large animals, we

administered to dog M57 a LV dose of 5.7×10^8 TU/kg, 44 μg p24/kg, approximately 40-fold lower in TU (and 12- to 24-fold lower in p24) than the above mentioned dose on a *per weight* basis. Since cFIX activity settled at approximately 0.1% of normal in this dog (about 50-fold lower than the above mentioned levels), this outcome suggests that our predictions based on the dose-response observed in the mouse model are reliable in the canine model as well. This also suggests that the TU dose is a better predictive factor than the particle dose, at least in this low-dose range. By process optimisation and transgene codon-optimisation we could administer to a second dog (O21) a LV dose 4-fold higher in TU/kg and only slightly higher in p24/kg. This dog achieved about 0.5-1% of normal FIX activity, approximately 10-fold higher than that achieved in the first dog, which can be explained by the higher dose and the co-cFIX transgene, which probably provided a 2.5-fold improvement in transgene expression. This also suggests that the 2- to 3-fold gain in potency observed in haemophilia B mice with the co-cFIX transgene has been recapitulated in the canine model. It has been recently reported that a short course of Dexamethasone administered around LV administration increases LV transduction of hepatocytes in mice²⁸⁷. We cannot exclude that the single-dose of Dexamethasone administered to this dog during LV infusion also helped hepatocyte transduction. By exploiting the Padua mutation which confers 5- to 10-fold hyperactivity to FIXa, we may now be able to achieve >5% of normal FIX activity in the canine model, following liver gene therapy with the co-cFIX-R338L transgene. By this additional 5- to 10-fold gain in potency we could reasonably decrease the effective LV dose to about 1×10^9 TU/kg, a target that we can comfortably reach with the current manufacturing technology for the treatment of haemophilia B dogs. During the cumulative 3.6-years period of observation of the two treated dogs only 3 out of 19 expected spontaneous bleedings occurred, indicating that the therapy provided a clinical benefit.

We observed transient fever and a mild self-limiting hepatic toxicity in both dogs receiving portal-vein administration of LV. This toxicity may be due to VSV-G-mediated fusion of transduced hepatocytes and subsequent death and may be decreased by using alternative envelope proteins such as gp64. Alternatively it may be due to an inflammatory response or mast-cell derived histamine release secondary to innate immune recognition and complement binding of LV particles. This toxicity did not

increase with a higher LV TU dose and may be mainly due to particles themselves. Rather, it was slightly decreased in the dog receiving anti-inflammatory and anti-histamine drugs, suggesting that there may be an important contribution of acute inflammation. The slight reduction in platelet counts is consistent with haemodilution due to the large amounts of liquid infused during the administration procedure. Haematocrit was also decreased for few days after the procedure, further confirming this hypothesis.

In one out of two dogs we observed an unexpected severe hypotensive reaction during LV infusion, probably due to a type-I anaphylactoid reaction to an unknown component of the LV batch. There were no major differences in the levels of protein or DNA contaminants or endotoxin between the infused batches. However, it is possible that the LV batch contained trace amounts of bovine serum albumin or penicillin derived from the supernatant of producer cells. Since haemophilia B dogs receive frequent plasma transfusions in response to spontaneous bleedings it is also possible that they are particularly sensitive to allergic reactions. This reaction was managed by a single-dose of an anti-inflammatory and a anti-histamine drug administered during the course of infusion. Pre-administration of analogous treatments before LV infusion may be proposed in the case of future LV administration to dogs or humans.

We observed a transient, rapidly declining rise in serum levels of TNF- α , IL-6 and IL-8. This is consistent with an inflammatory response secondary to a type-I IFN response due to the recognition of the viral RNA by pDCs, as observed in previous studies conducted in mice (see also 1.4.1)²⁴¹. The administration of anti-inflammatory drugs as proposed may also contain this increase in serum cytokine levels.

Overall our results show that LV-mediated liver gene therapy is feasible in dogs and can provide long-term cFIX expression and activity up to 0.5-1% of normal and therapeutic benefit with a manageable self-limiting acute toxicity in the absence of long-term toxicities and without eliciting anti-transgene immune responses.

5.2 Codon-optimised hyper-active FIX transgene

Increasing the potency of gene transfer vectors is crucial for gene therapy, as much as for any “conventional” drug, as it results in an improved therapeutic index. Indeed lowering the dose necessary to achieve a therapeutic response decreases dose-dependent

toxicities and immunogenicity. At the same time it reduces the costs and hurdles associated with manufacturing, ultimately resulting in a more comfortable therapeutic window of the proposed gene therapy. Towards this goal, we have undertaken efforts to improve expression and activity of the FIX transgene *per* vector copy.

Nature has evolved codon usage in genes to fine tune translation rate and adapt protein quantities in cells^{288, 289}. We have exploited a straightforward strategy aimed at increasing vector potency: the modification of transgene sequences in order to maximise the output of the transgene product from a single expression unit. There is growing evidence that codon-usage and *cis*-acting sequences optimisation can increase the potency of gene transfer vectors^{25, 290, 291}. A 3- to 4-fold increase in hFIX expression levels has been obtained by codon-usage optimisation of the hFIX transgene upon liver gene therapy with scAAV vectors in wt and haemophilia B mice²⁹². We exploited a co-cFIX transgene to increase the potency of LV for liver-directed haemophilia B gene therapy and showed a robust 2- to 3-fold increase in cFIX expression over the wt transgene in 3 different experiments in haemophilia B mice and in one haemophilia B dog.

Increasing the transgene product output from a single expression cassette risks circumventing miR-142 regulation and may lead to transgene expression in APCs and anti-transgene immune responses. Directing hepatocytes to produce high quantities of the transgenic protein may saturate the translation or post-translational modification machinery and eventually expose cells to toxicity. Moreover, it appears that changing codons in genes can alter the translation velocity thus impairing proper folding and/or post-translational modifications²⁶⁷. Therefore, it is necessary to be cautious when generating codon-optimised transgenes and it is essential to carefully evaluate them in pre-clinical models. We showed long-lasting FIX activity in treated mice without any evident decrease of specific activity nor increased immunogenicity nor counterselection of transduced hepatocytes and decrease of FIX over time. This suggests that none of the previous concerns has occurred.

To further increase vector potency we exploited the naturally occurring single-amino acid R338L (Padua) mutation in FIX, associated with clotting hyperactivity and thrombophilia²⁶⁸. Engineered FIX variants with higher catalytic activity or augmented bioavailability have been previously reported and adopted in gene therapies studies

involving Ads and AAV vectors^{269, 293, 294}. These variants carry the same amino acidic substitution at residue 338, which is critical for substrate binding, mutations in other domains of the protein or combinations of multiple mutations. None of the previously described single mutations in the FIX reaches the same gain in catalytic activity conferred by the Padua mutation, to our knowledge. We showed that the corresponding mutation in the cFIX results in increased enzyme specific activity 5- to 10-fold and allows the use of lower LV doses to achieve sustained therapeutic efficacy in haemophilia B mice. We show for the first time the superior performance of FIX-R338L in achieving haemostasis *in vivo* in haemophilia B mice by a quantitative tail-bleeding assay. We also show that this mutation does not impair the tolerogenic outcome of LV-mediated liver gene therapy, as treated mice were unresponsive to immune challenge with the wt protein.

While FIX-R338L causes thrombophilia in an individual with normal FIX levels, it is highly unlikely that this would also occur in a haemophilic background. Clinical evidence indicate that FIX activity up to 300-400% of normal are tolerated and do not result in an increased risk of thrombosis. Indeed the mother of the patient in which the R338L mutation was found, who is heterozygous for the mutation, has not undergone thrombotic events²⁶⁸. Moreover this mutation causes an increased catalytic activity of FIXa and not non-specific zymogen activation²⁶⁸. Our extensive *post-mortem* pathological analysis of the organs of treated mice and the absence of D-Dimers elevation in the circulation suggest that thrombotic risk was not increased upon delivery of FIX-R338L transgene. However, a more extensive evaluation of the safety of delivering such hyper-functional FIX transgene will be appropriate before considering clinical translation. Several mouse models sensitive to vascular thrombosis have been described and may serve this purpose. These models include the ferric chloride injury model and the FV Leiden mutation mouse model^{271, 295}.

By the novel combination of codon optimisation with the hyper-activating FIX-R338L mutation we achieved an unprecedented robust >15-fold gain in potency and therefore provide a promising strategy to improve the efficacy, feasibility and safety of haemophilia gene therapy with LVs as well as other vector platforms.

5.3 Baculovirus gp64-pseudotyped LV for liver gene therapy

When directly injected into the circulation, LV particles can be inactivated by complement-mediated lysis due to the binding of high-affinity specific or non-specific low affinity pre-existing antibodies to the envelope protein or other membrane proteins carried over on the viral particles from producer cells^{263, 264, 296}. In addition, antibody binding opsonises viral particles and favours both their elimination through phagocytosis and inflammatory responses. It has been reported that RVs produced in cells of non-human origin carry on the surface galactosyl (α 1-3)galactosyl terminal glycosidic epitopes that are efficiently recognized by specific human and primate antibodies and there are inactivated by serum more than 100-fold *in vitro*. Moreover their systemic stability is drastically reduced upon i.v. injection in chimpanzees²⁹⁶. Other factors, particularly the envelope glycoprotein, also contribute in determining complement sensitivity. It has been shown that VSV-G-pseudotyped LV are sensitive to inactivation in serum from humans and primates²⁶³.

By using an *in vitro* assay of LV infectivity reduction by the heat-labile serum complement system, we showed that canine serum can inactivate VSV-G-pseudotyped reporter LVs 3- to 5-fold and inter-individual variability was present. We reasoned that this low-level inactivation would not compromise direct *in vivo* administration to dogs. Although all treated dogs were chosen among 3 candidates, on the basis of the results of the *in vitro* inactivation assay, serum of these dogs was still able to inactivate LVs 2- to 4-fold. Since the transgene levels obtained in these dogs were in line with predictions based the dose infused and the dose-response observed in mice, this indicates that the level of inactivation observed in the *in vitro* assay did not impair liver transduction. The inactivation observed in serum derived from two human donors was higher (approximately 10-fold). The predictive value of this *in vitro* assay is questionable and *in vivo* studies may be more appropriate to assess the impact of pre-existing natural immunity on vector biodistribution and transduction. The incorporation of complement regulatory proteins on the surface of vector particles has been shown to improve complement-resistance and stability and it may also decrease innate immunity activation^{264, 297, 298}, thus it may be considered for clinical translation of LV-mediated liver gene therapy.

In agreement with previous publications^{264, 272}, we showed that LVs pseudotyped with the baculovirus gp64 envelope protein are more resistant to inactivation by human and canine serum. The use of gp64 would also improve LV tropism for hepatocytes, by limiting transduction of hematopoietic-lineage cells and it has been shown to further reduce anti-transgene immune responses²⁴⁴. This may also reduce toxicity due to interactions with the complement system and hematopoietic-lineage cells. Restricting the spectrum of transduced cell types may also reduce safety concerns and the risk of germline transmission. Large-scale manufacturing, however, still needs to be adapted for this new envelope. On the other hand, since VSV-G expression is cytotoxic for producer cells, the use of gp64 may facilitate the development of stable packaging cell lines for an efficient scale-up of vector production. We showed that gp64.LVs can be produced to high titre and that they mediate efficient liver-directed gene therapy and expression of hFIX in haemophilia B mice to levels comparable to those obtained with matched doses of VSV-G pseudotyped LVs. For these reasons gp64 may provide a better alternative to VSV-G as a LV pseudotype for clinical translation of liver gene therapy. However, VSV-G pseudotyped LVs currently remain the preferred option, unless a robust process will be put in place for gp64-pseudotyped LV and complement sensitivity will represent a serious concern during future clinical development.

5.4 Proteasome inhibition in LV-mediated liver gene therapy

LVs efficiently transduce hepatocytes *in vitro*. However the tight KC network lining liver sinusoids and LSECs represent barriers for LVs approaching hepatocytes *in vivo*. In addition to these physical barriers, there are biological defences interfering with LV transduction of hepatocytes. Thus finding ways to circumvent these blocks can improve hepatocyte transduction.

It has been previously reported that KCs restrain hepatocyte transduction by LV²⁹⁹. Here we present the first comprehensive quantitative analysis of the biodistribution of LV within the liver cell subpopulations at steady state, without confounding factors deriving from anti-transgene immune responses. Our data provide evidence that KCs represent the major site of LV accumulation *in vivo* and likely an important impediment to efficient LV transduction of hepatocytes. KCs show very high LV copies *per cell*, although accounting for only about 6% of the total liver cells. Indeed up to 70% of LV

DNA is found in these cells at steady state. However this percentage varies with different LV doses and decreases at increasing vector doses. This suggests that hepatocyte transduction becomes more efficient above a “threshold” LV dose at the expense of KCs transduction, when it is reasonable to speculate that “saturation” of transduction occurs or the cells with highest VCN undergo cell death. It is currently unknown whether this “saturation” is due to decreased KC phagocytic activity or simply to an increased likelihood for the LV particles to get access to hepatocytes. In any case, this phenomenon is consistent with the observed non-linear correlation between the LV doses and both transgene expression in hepatocytes and the VCN measured on whole liver. Furthermore it indicates that the whole liver VCN is not the best readout of the extent of hepatocyte transduction as it can mask a different contribution by PCs and nPCs. Instead, transgene expression in hepatocytes show a perfectly linear correlation with the VCN measured on the population enriched in PCs. Similar findings have been observed in the case of liver transduction with Ads, as KCs depletion improves their ability to transduce hepatocytes, which also show a non-linear correlation with the administered vector dose³⁰⁰⁻³⁰². Our analysis can be applied to understand how different treatments (such as KCs depletions or agents interfering with their function) as well as different LV pseudotypes affect the distribution of LV DNA within the liver cell subpopulations, in order to find ways to improve hepatocyte transduction. Of note, the LV doses used in our dog studies would probably fall below the threshold for more efficient hepatocyte transduction, which would likely strongly benefit from pre-treatments such as bortezomib administration.

Some cell types and host species can restrict LV transduction to a different extent. It has been previously shown that proteasome inhibition improves LV transduction in several types of stem cells²⁷⁸, possibly by impairing cytosolic degradation of the uptaken vector particles that undergo uncoating, and thus enhancing their nuclear translocation. Here we show that proteasome inhibition with Bortezomib increases LV transduction of hepatocytes both *in vitro* and *in vivo* by a poorly understood mechanism. The increase in hepatocyte transduction observed *in vitro* indicates a cell-autonomous (hepatocyte-dependent) mechanism, which may conceivably contribute to the observed increase in hepatocyte-derived hFIX expression after liver transduction *in vivo*. However, since the whole liver VCN was not increased, this prompted us to investigate

whether there was an increase of the VCN in hepatocytes at the expenses of the VCN in nPCs. We found a substantial decrease in VCN in nPCs and KCs in mice pre-treated with Bortezomib as compared to controls. However this was not accompanied by a detectable increase in VCN in PCs. Unfortunately our separation method does not yield purification of hepatocytes, but rather enrichment for these cells. Given the high VCN in KCs, as little as 1-2% of these cells contaminating PC preparations can affect the measured VCN in PCs and since pre-treatment with Bortezomib resulted in a 2-fold decrease in the VCN in KCs we speculate that this masked the complementary increase in VCN in PCs. As an alternative explanation, we cannot exclude that proteasome inhibition has altered the integration profile of LV in hepatocytes leading to an improved transgene expression without increased VCN in hepatocytes in mice³⁰³.

The decrease in VCN in KCs observed after Bortezomib pre-treatment can be explained by an impaired phagocytic activity at the time of LV transduction or by an enhancement of toxicity in highly transduced KCs and subsequent replacement from non-transduced progenitors, or both. Bortezomib has anti-inflammatory properties, due to inhibition of the NF- κ B pathway^{275, 304} and has been reported to inhibit osteoclast activity and KCs activation^{276, 305}. Thus these data may play in favour of the first hypothesis. While acute toxicity on macrophages has not been reported, it is possible that temporary inhibition of proteasome activity resulted in protein toxicity in these cells that have high phagocytic activity.

It has been shown that type-I IFNs disfavour hepatocyte transduction, by inducing an anti-viral state and potentially up-regulating restriction factors^{241, 287}. In an attempt to dampen these effects Agudo et al. have shown that treatment with the anti-inflammatory dexamethasone increases hepatocyte transduction by LVs and decreases KCs transduction in mice²⁸⁷. Our data show that Bortezomib increases hepatocyte transduction also in mice defective for type-I IFNs signalling (*Ifnar1*^{-/-}), thus suggesting that Bortezomib-mediated increase in hepatocyte transduction is not exclusively due to inhibition of this pathway. However, since the increase in hepatocyte transduction was more pronounced in *Ifnar1*^{-/-} than haemophilia B mice, it is possible that there are additive or synergistic effects and it would be interesting to evaluate whether Bortezomib and dexamethasone in combination have a stronger effect on LV transduction of hepatocytes.

Overall our data indicate that a single administration of the proteasome inhibitor Bortezomib prior to LV administration increases hepatocyte transduction by 2- to 3-fold. This is most probably obtained through a combination of both a cell-autonomous mechanism directly inhibiting restrictions and proteasome-mediated degradation of LV capsids, and a non-cell autonomous (hepatocyte-independent) mechanism interfering with KCs transduction. Since clinical experience indicates that Bortezomib toxicities are mostly due to long-term exposure to the drug, this represents a viable strategy to improve the potency of LV-mediated liver gene therapy.

5.5 LV-mediated liver gene therapy in mice with pre-existing FIX inhibitors

The major side-effect of FIX replacement therapy, development of inhibitors to the infused concentrate, is the main threat to the health of haemophilic patients and consequently the goal of intense research. Here we show that LV-mediated liver gene therapy has the potential to revert pre-existing anti-FIX humoral immunity and eradicate inhibitors in mice both following a relatively early and a relatively late intervention after the development of anti-FIX antibodies in haemophilia B mice. Once the anti-FIX antibody response has declined, liver gene therapy can provide sustained endogenous FIX expression to therapeutic levels. These effects are accompanied by a significant decrease in anti-FIX antibodies secreting cells in the lymphnodes of mice treated with gene therapy as compared to controls, although a complete evaluation of the B-cell response in other lymphoid organs will need to be performed. We also report almost complete disappearance of FIX-specific B_{MEM} in mice treated with gene therapy 10 weeks after FIX immunisation, once the anti-FIX antibody response is fully mature.

We postulate that the mechanism by which this occurs is not dissimilar to that invoked to explain eradication of inhibitors by ITI. It has been reported that sustained exposure to high levels of antigen induces anergy or apoptosis of B_{MEM} in haemophilia A mice in which an anti-FVIII antibody response was induced²⁸². Liver gene therapy can also exert this effect, with the accompanying benefit of inducing transgene-specific Tregs which keep under control CTL responses and T-helper mediated stimulation and maturation of antigen-specific B lymphocytes. Once the B_{MEM} have been shut down, the

antibody response gradually declines, as plasma cells turnover. This may be further accelerated by additional treatments depleting the remaining plasma cells.

Gene therapy has been proposed as an attractive therapeutic strategy also for inhibitors-positive haemophilic patients, for which the current treatment is unsatisfactory and may thus provide a more favourable alternative³⁰⁶. Gene therapy may be an alternative treatment to ITI to eradicate inhibitors and at the same time provide sustained endogenous expression of the clotting factor. It has been recently reported that liver gene therapy with AAV vectors can establish cFVIII expression (by the “dual vector” approach) in haemophilia A dogs with pre-existing anti-FVIII antibodies³⁰⁷. This therapy also resulted in a gradual decline of anti-FVIII antibodies titre in treated dogs over the course of 1 year. We show eradication of FIX inhibitors by liver gene therapy in haemophilia B mice. Although the frequency of inhibitor development is lower in haemophilia B patients, clinical experience with ITI is limited and there is a higher risk of anaphylactic reactions in these patients undergoing ITI^{98, 99}. It is possible that haemophilia B mice with anti-FIX antibodies have experienced anaphylaxis or immune complex-mediated hypersensitivity upon high-dose i.v. injections of the recombinant FIX protein. This has been previously reported for FIX injections as well as other therapeutic proteins in mice with pre-existing antibodies^{281, 308}. Further investigations will be necessary to elucidate this aspect. Although most of the FIX immunised haemophilia B mice did not develop high-titre inhibitors, our data suggest that liver gene therapy may be more effective and safer than ITI in eradicating FIX inhibitors and can provide therapeutic FIX levels in haemophilia B mice with pre-existing anti-FIX immunity.

Our results demonstrate that liver gene therapy can establish tolerance not only in a naïve immune system, but also in an already antigen-primed immune system and may candidate liver gene therapy as a therapeutic option also to revert autoimmune diseases.

5.6 IDLVs for liver gene therapy

IDLVs are emerging as an attractive platform for transgene expression for several purposes³⁰⁹. This platform harnesses the pantropism and proficiency of LV transduction without relying on integration and permanent modification of the cellular genome. Here we show that IDLVs can be used to express transgenes for a window of time in the

liver, provided that expression is stringently targeted to hepatocytes using transcriptional and miR-142-mediated regulation. Whereas the IDLV expression efficiency is lower than that observed for ICLV, expression levels suffice to induce immune tolerance. Moreover, the non-integrating feature of the platform provides for important safety advantages due to the low risk of genotoxicity and the reversibility of transgene expression.

The declining transgene expression of IDLV in proliferating cells has been mostly ascribed to the progressive loss of episomal DNA from the cell nucleus at mitosis, as compared to the stably integrated provirus of ICLV. Here, we reliably quantified the content of reverse transcribed vector genomes and the transgene expression level in transduced dividing or quiescent cells, and show that the expression efficiency *per* genome copy is significantly lower for IDLV than for ICLV in hepatocytes. This may be due to diffusion of the episomes to nuclear areas that are not involved in active transcription, inefficient chromatin deposition, or enrichment with histone modifications typical of transcriptionally silenced chromatin³¹⁰. This is in contrast to what has been reported in other quiescent tissues, such as the retina and the brain, and may indicate tissue-specific factors affecting the expression and stability of episomal IDLV. It is conceivable that incorporation of additional genomic elements into the IDLV backbone may improve their expression proficiency and nuclear stability.

We show that background integration of IDLV occurs *in vivo* by mechanisms incompatible with residual activity of the mutant integrase, as they often present deletions in the LTR ends and lack the typical flanking genomic repeats at the insertion site. These events may be mediated by non-homologous end joining of linear episomes to sites of chromosomal breakage³¹¹. Though we cannot rule out a contribution to the observed sustained FIX expression by this integrated IDLV, the significant decline of FIX expression by inducing hepatocyte proliferation after partial hepatectomy strongly suggests that *in vivo* expression is mostly mediated by the episomal forms.

The significantly lower and temporary hepatocyte expression of IDLV as compared to ICLV represents a limiting factor for their application to stable therapeutic gene replacement in the liver, at least in the current design. However, IDLV may be considered whenever reversible gene transfer is preferable, such as when testing a new gene therapy approach, especially if the clinical setting imposes high safety bars in the

face of existing treatment options, as in haemophilia, or when the biological effects of gene-based delivery are difficult to predict or may entail substantial toxicity. Here we demonstrate the therapeutic potential of inducing a prolonged window of FIX expression in the plasma of haemophilic mice. Apparently, FIX expression was more prolonged than GFP expression following hepatic transduction with IDLV. This possibly reflects the higher vector doses used and/or differences in detection limits of the assays used to quantify expression of the respective transgene products. IDLV may be used for hepatic expression of therapeutic proteins, such as IFN or other cytokines, in chronic viral hepatitis or hepatic tumours, in which gene-based delivery may provide therapeutic concentrations of the factor at the disease site with limited systemic exposure and only for a defined window of time^{312, 313}. Alternatively IDLV may be exploited for the temporary expression of site-specific nucleases to perform gene targeting in hepatocytes.

Hepatic gene transfer has been associated with the induction of immunological tolerance to the transgene product using several vector platforms (see 1.1.8.1)^{54, 274}. In contrast to other viral vectors used in gene therapy, most subjects are immunologically naïve to the IDLV vector components thus it is unlikely that IDLV-transduced hepatocytes would be recognized by vector-specific CTLs. Here we demonstrate a major accompanying benefit of IDLV-mediated hepatocyte-targeted transgene expression, i.e. the induction of transgene-specific Tregs and active tolerance to the transgene product. Most importantly, this response may extend beyond the duration of vector-mediated transgene expression. It has been shown that transgene persistence is not a strict requirement for the maintenance of immune tolerance³¹⁴. The *in vivo* induction of antigen-specific Tregs by hepatocyte-targeted IDLV-mediated expression may well represent their most attractive feature to date and provides an intriguing contrast to the immunogenic nature of non-regulated antigen expression by IDLV delivery, which is currently being explored for the design of improved viral-based vaccines^{315, 316}. Although IDLV are less efficient at expressing the transgene in hepatocytes as compared to their integrating counterparts, they are equally efficient at inducing transgene-specific tolerance, suggesting that the pattern and not the levels of transgene expression plays a crucial role in directing the immune system response in this setting. It is likely that different mechanisms are involved in tolerance induction

towards intracellular or secreted antigens and further studies will be required to better elucidate this aspect.

A broad application of hepatocyte-targeted expression by ICLVs for immune modulation is currently limited by the concerns associated with integration in the target cell genome. IDLVs are advantageous for this purpose and could be exploited in “inverse vaccination” strategies to tolerise individuals to protein replacement therapies by preventing induction of neutralizing antibodies, which represent one of the major hurdles of this treatment³¹⁷. Moreover these strategies may be adopted to prevent or revert the development of autoimmune diseases as well³¹⁸.

5.7 Oncogenic risk in LV-mediated liver gene therapy

As we found that integrations is preferable for stable efficient liver gene transfer, we set out to assess the oncogenic risk associated with LV integration in hepatocytes. Whereas liver gene therapy by miR-142 regulated LVs did not cause detectably adverse events in any of the treated haemophilic mice, concerns remain regarding the long-term risk of vector insertional mutagenesis and studies in more stringent models are desirable. Most studies of vector genotoxicity have been performed in the hematopoietic system, thus very little is known about this risk in other tissues, such as the liver. Sensitive *ad hoc* murine models that are prone to develop HCC have been established (Ranzani et al., Nature Methods, in press). One model is characterized by the genetic deficiency of a tumour suppressor pathway (*Cdkn2a*^{-/-}), the other is based on wt mice in which HCC induction is promoted (but not initiated) by repeated injections of carbon tetrachloride (CCl₄). We have administered ET.FIX.142T LVs in the therapeutic configuration in parallel with a specifically tailored genotoxic LV design (used as a positive control for insertional mutagenesis) into these mice at neonatal age. While the latter vector reproducibly induced HCC, we have not detected any liver tumour by our therapeutic LV. We then retrieved 9,215 unique insertion sites from the treated mice. Whereas integrations of the genotoxic LV were enriched at previously validated HCC oncogenes, indicating positive selection of cancer-causing events, the therapeutic LV did not show an integration bias for these genes. Moreover, we did not detect any evidence of selection for the therapeutic LV insertions. These data indicate a low risk of insertional oncogenesis by LV-mediated liver gene therapy.

5.8 Conclusions

LVs are attractive tools for liver gene therapy, by virtue of their ability to stably integrate their genome into host cells, which renders them applicable in young patients in which hepatocyte turnover is high. Moreover, in contrast to other vector platforms such as Ads and AAV vectors, there is no pre-existing humoral or cellular immunity against vector components in most humans.

We have undertaken a comprehensive study aimed at developing LV-mediated liver gene therapy, by improving its potency, strengthening and broadening its possible applications, utilising both small and large animal models. We performed the first LV-mediated liver gene therapy in haemophilia B dogs and show long-term transgene expression and some therapeutic benefit in two dogs with only mild acute hepatic toxicity and without long-term toxicities nor induction of anti-transgene immune responses. Our study reveals that one major issue limiting therapeutic efficacy is that *in vivo* biodistribution among liver cell populations limits hepatocyte transduction especially at low administered doses. We show that the use of optimised expression cassettes and hyperactive transgenes can increase the potency of LVs and that pharmacological pre-treatments help getting access to hepatocytes, thus bringing therapeutic doses to levels comfortably attainable by the current manufacturing technology and overall improving the therapeutic index of this therapy. Improvements in the production and purification of large-scale LV preparations as well as the current development of stable packaging cell lines will further facilitate large-animal testing and clinical translation. More extensive pre-clinical studies aimed at evaluating biodistribution and the risk of germline transmission will be required before clinical translation. We provide evidence that liver gene therapy can establish long-term transgene expression and tolerance in mice with pre-existing anti-transgene immunity. We report a comprehensive evaluation of IDLVs for liver gene therapy and show that, while not optimal for stable gene replacement therapy in their current design, they may represent a valuable strategy to induce stable antigen-specific tolerance by transient gene transfer and offer a treatment for immune-mediated diseases. On the other hand, since LV integration is preferable for efficient stable liver gene transfer, we stringently assessed the risk of oncogenesis associated to LV integration in *ad hoc* mouse models

that are sensitised to develop HCC and found no detectable increase in carcinogenesis upon liver gene therapy with LVs.

Gene therapy has now restored vision in blind patients, immune defences in immune-deficient patients, blood in anaemic patients and coagulation in haemophilic patients and more has yet to come^{19, 20, 22, 23, 25}. *Ex vivo* gene therapy with HSPCs has entered clinical experimentation since more than a decade and there is great promise for future therapies. Liver gene therapy faced other challenges in clinical translation, mainly due to the hurdles associated with the immune consequences of direct *in vivo* administration. However it has steadily progressed to human testing. AAV vectors have now shown remarkable clinical benefits in haemophilia B patients upon liver gene therapy. LVs may well complement other available vectors to address the different challenges posed by the presentation of haemophilia and its complications in different patients and clinical conditions and may conceivably offer a therapeutic option for lysosomal and metabolic diseases.

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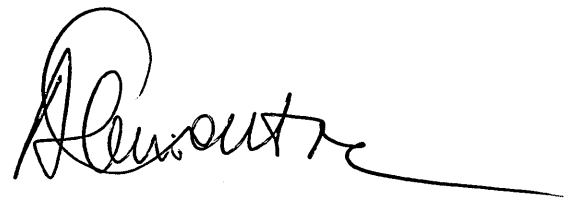
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A handwritten signature in black ink, appearing to read 'A. Coutant', with a long horizontal flourish extending to the right.

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